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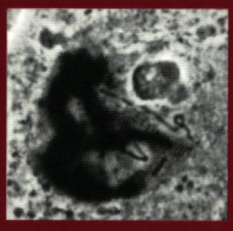
ANATOMY

of a lampbrush loop-forming male fertility gene on the Y chromosome of Drosophila hydei



Ron Hochstenbach

ANATOMY OF A LAMPBRUSH LOOP-FORMING MALE FERTILITY GENE ON THE *Y* CHROMOSOME OF *DROSOPHILA HYDEI*

ANATOMY OF A LAMPBRUSH LOOP-FORMING MALE FERTILITY GENE ON THE *Y* CHROMOSOME OF *DROSOPHILA HYDEI*

een wetenschappelijke proeve
op het gebied van de Natuurwetenschappen

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A hair perhaps divides the false and true.

Rubáiyát
Omar Khayyám

CHAPTER 1

The lampbrush loop-forming male fertility genes on the *Y* chromosome of *Drosophila*-77 years of mystery

1 INTRODUCTION

The combined results of three papers, two of them published in 1916 by Bridges, and one in 1961 by Meyer, Hess and Beermann, have laid the foundation for all investigations into the molecular structure and the biological function of the lampbrush loop-forming male fertility genes on the *Y* chromosome of *Drosophila*.

Bridges provided the first experimental evidence for the presence of genes on the *Y* chromosome of *D. melanogaster* that are essential for male fertility. Meyer et al. described the lampbrush loop-like structures in the nuclei of primary spermatocytes, and they concluded from their observations that the loops are formed by genes on the *Y* chromosome.

During the last 77 years, these mysterious genes have inspired numerous genetic, cytological, ultrastructural, immunological and molecular studies. The genes were found to display some fascinating, special properties, that are not shared with other genes of *Drosophila*: they are much larger than even the largest protein coding gene, they have a very high mutation frequency, they seem to be composed only of repetitive DNA sequences, and they are the only genes in *Drosophila* that form lampbrush loop pairs.

In this chapter, the available information on the structure and the function of the lampbrush loop-forming fertility genes will be summarized. Especially the formal genetic and cytogenetic aspects of the loop-forming genes will be extensively discussed, as these cannot be separated from their biological functions and the underlying molecular mechanisms. Almost all research has been done on either *D. melanogaster* or *D. hydei*, but it is assumed that the functions of the *Y* chromosomal fertility genes are conserved throughout the *Drosophilidae*. Therefore, evolutionary aspects will be included wherever possible.

This large body of literature contains several hypotheses about the molecular basis of the function of the loop-forming fertility genes. The two major hypotheses that have been recently put forward are the following: the loop-forming fertility genes function by **coding for proteins** that are required for spermatogenesis (Leoncini 1977; Goldstein et al. 1982; Gepner and Hays 1993). They may also function by the **binding of proteins** that are encoded by genes on other chromosomes (Hennig 1985; Gatti and Pimpinelli 1992).

It will be shown in this chapter that one reason for our lack of understanding of the function of the loop-forming genes is our fragmentary knowledge of the organization of the DNA sequences within the loop-forming transcription units. In an effort to fill up this knowledge gap, the major part of this thesis deals with the molecular structure of the *Nooses*, the lampbrush loop pair formed by fertility gene *Q* on the *Y* chromosome of *Drosophila hydei*. Because the lampbrush loops are expressed only during the postreplicative meiotic prophase, when the DNA content of the cell is 4C, they will be referred to as "loop pairs" rather than "loops". The work described in this thesis was conducted to address the following questions:

(i) What is the nature of the DNA sequences that are transcribed in the *Nooses* loop pair?

(ii) How are these sequences organized within the loop?

(iii) Is it possible to reconstruct the evolutionary history of the loop?

(iv) What is the potential significance of these sequences for the function of fertility gene *Q* ?

(v) Can we derive the function of the lampbrush loops from these data?

2 GENETIC AND CYTOGENETIC MAPPING OF THE FERTILITY GENES

2.1 The discovery of the fertility genes

In 1916, Bridges observed exceptional progeny classes in the offspring from a cross of a wild-type *Drosophila melanogaster* male and a female that was homozygous for the *X*-linked eye colour mutation *white*. In addition to white-eyed sons and red-eyed daughters, the classes expected from a regular Mendelian segregation, he also found, at a frequency of approximately 0.05%, exceptional sons, displaying the bright red eye colour of their father, and exceptional daughters, displaying the white eye colour of their mother. Bridges showed that the exceptional males carried an *X* chromosome, which they must have inherited from their father, but no *Y* chromosome. Males of this constitution were called *XO* males. The exceptional daughters had the normal number of two *X* chromosomes, which they both inherited from their mother, but they also had their father's *Y* chromosome. Bridges concluded that the exceptional offspring classes resulted from a failure of the mother's two *X* chromosomes to segregate during the first meiotic division, a phenomenon called primary non-disjunction.

The interpretation of this experiment is crucial for the understanding of the nature of the genes located on the *Y* chromosome. A first conclusion is that there are no genes on the *Y* that are essential for male viability, since *XO* males develop and live normally. In 1982, Miklos showed that the average time needed by *XO* males for completing embryonic development is only 1% different from the time needed by *XY* males. Second, the experiment showed that the *Y* chromosome had nothing to do with sex determination, since the males phenotype is established without the *Y* chromosome, and the female phenotype is not affected by the presence of an *Y* chromosome. However, the experiments also revealed that the *Y* chromosome does carry some genes. The patroclinous, red-eyed sons, although apparently normal in their somatic phenotype, were unable to produce any progeny. This result was the first evidence for the location of genes on the *Y* chromosome that are essential for male fertility.

2.2 Fertility genes of *D. melanogaster*

There are several reasons why the mapping of the *Y* chromosomal fertility genes has been more difficult than that of most genes on the other chromosomes. The *Y* chromosome normally does not carry phenotypic markers. Commonly used modified *Y* chromosomes carrying visible markers at their terminal ends are described by Lindsley and Zimm (1987) and Ashburner (1989). Conventional gene mapping by determining genetic distances from crossing-over frequencies between homologous chromosomes is not possible, as there is only one *Y* chromosome present in males. In addition, there is no recombination in *Drosophila* males (Morgan 1912, 1914). Thus, the mapping of *Y*-linked fertility genes had to rely on complementation tests performed by combining two different *Y* chromosomes, or segments thereof,

in male flies (see below). Since the *Y* chromosome is, like most of the other heterochromatin, underreplicated in polytene cells (Heitz 1933), the mapping of breakpoints of deletions, inversions and translocations was possible only in metaphase chromosomes. The application of chromosome banding techniques, allowing some longitudinal differentiation of the *Y* chromosome (Fig. 1), enhanced the sensitivity of this technique (Gatti et al. 1976; Pimpinelli et al. 1976). The *Y* chromosome of *D. melanogaster* has a length of about 40 000 kb (Pimpinelli et al. 1978). Approximately 25 cytological landmarks can be distinghuished in a metaphase *Y* chromosome, and therefore, the resolution level does not exceed 1000-2000 kb of DNA. This is two orders of magnitude lower than the 10 kb resolution level possible in polytene chromosomes (Rykowski et al. 1988).

Following the pioneering work of Bridges (1916), Stern (1929) used translocations of either the long or the short arm of the *Y* chromosome and the *X* chromosome, to show that both arms of the *Y* chromosome harbor fertility genes. Neuhaus (1939) used unmarked, X-ray-induced fragments of the *Y* chromosome that were translocated to autosome *4*. Complementation tests between the different fragments revealed four fertility genes on the long arm and five on the short arm. By performing complementation tests between X-ray-induced male-sterile mutations on a marked *Y* chromosome, Brosseau (1960) was able to establish the linear order of five complementation groups on the long arm, and two on the short arm.

Since then, several other investigators have, using different marked *Y* chromosomes, confirmed Brosseau's results, with the only exception that the existence of one of the complementation groups on the long arm could not be verified. Hazelrigg et al. (1982) used X-rays and Kennison (1983) used either Ethyl Methane Sulphonate (EMS) or γ-rays for the induction of male-sterile mutations. These mutations were obtained at high frequencies (2.5-5% of the treated chromosomes). In the majority of cases, male sterility was associated with a chromosomal rearrangement involving the *Y* chromosome. Gatti and Pimpinelli (1983) used the X-ray induced *Y*-autosome translocations (*T(Y;A)*s) generated by Lindsley et al. (1972), and male-sterile mutations, newly induced by γ-ray irradation. In all these investigations, the complementation tests of the different *Y* chromosomes consistently revealed four complementation groups on the long arm (which, from distal to proximal, are named *kl-5*, *kl-3*, *kl-2* and *kl-1*), and two on the short arm (named *ks-1* and *ks-2*, with *ks-1* more proximally), as is discussed subsequently.

Kennison (1981) applied the method of segmental aneuploidy (Lindsley et al. 1972) for the mapping of the fertility genes. He generated translocations of the *Y* chromosome to the proximal heterochromatin of the *X* chromosome, with the breakpoint in the *Y* not causing the sterility. By systematically testing these *T(X;Y)*s with *Y* chromosomes carrying deletions, he identified four regions on the long arm and two on the short arm that were essential for fertility.

The definitions of the different complementation groups by Kennison (1983) were based on at least 3, and in some cases even 12 newly induced noncomplementing mutations on cytologically normal *Y* chromosomes, *i.e* there was not a single case of interallelic complementation. In contrast, Williamson (1970, 1972) had reported intragenic complementation within several of the complementation groups originally identified by Brosseau (1960). However, these findings could not be reproduced

by other investigators (Hazelrigg et al. 1982; Kennison 1983; Gatti and Pimpinelli 1983). Hazelrigg et al. (1982) showed that several of the original Brosseau stocks had suffered from genetic breakdown, and that other stocks were mislabeled, providing an explanation for the aberrant observations of Williamson.

In summary, there are six complementation groups, each of them identifying one genetic function that is required for male fertility. This function can be destroyed by a break in the *Y* chromosome, but also cytologically normal *Y* chromosomes can carry male-sterile mutations (Gatti and Pimpinelli 1983).

The cytological locations of the complementation groups were determined by the mapping of breakpoints of *Y* chromosomal rearrangements causing male sterility on banded (pro)metaphase chromosomes from larval brains (Kennison 1981; Hazelrigg et al. 1982; Gatti and Pimpinelli 1983). The breakpoints that disrupted the same fertility gene (*i.e.* failed to complement a mutation or a deficiency of the fertility gene) had a highly nonrandom distribution and were clustered in four regions on the long arm, and two on the short arm (Fig. 1). As estimated from the location of the breakpoints of the sterile noncomplementing rearrangements, the physical sizes of *kl-5*, *kl-3* and *ks-1* were estimated at 4000 kb of DNA (reviewed by Gatti and Pimpinelli 1992). The localization studies further implied that large regions of the *Y* chromosome did not carry genetic functions required for fertility.

Nicoletti and Lindsley (1960) and Lindsley et al. (1972) observed that many *T(Y;A)*s are sterile, which was interpreted in favour of an inactivation of *Y*-linked fertility genes by long-range position effects due to their abnormal juxtaposition to euchromatin. Indeed, the displacement of wild-type genes from their normal position within the centromere-associated heterochromatin of chromosome *2* to euchromatic positions frequently affects their function. This has been most clearly shown for the gene *light* (Wakimoto and Hearn 1990) and for the gene *rolled* (Eberl et al. 1993) of *D. melanogaster*. Therefore, the sterilizing breakpoint in *T(Y;A)*s is not necessarily within the fertility gene, leading to overestimations of gene size. Also in the case of *T(X;Y)*s it cannot be excluded that sterilizing breakpoints are not within a fertility gene.

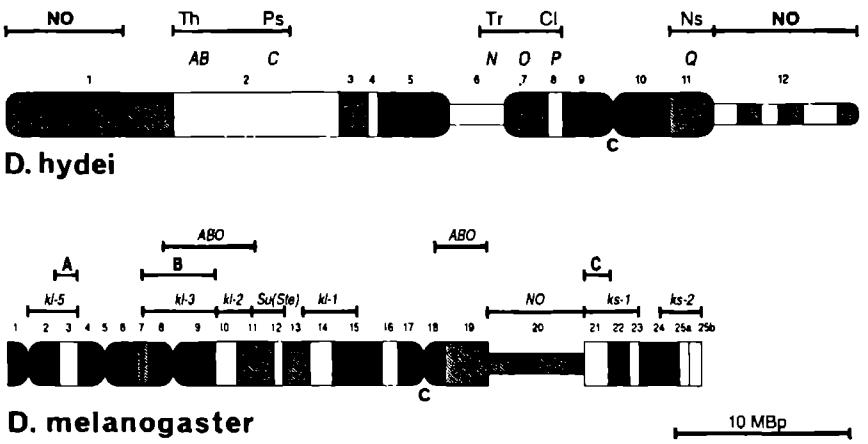
Although the extent of this problem is unknown, the mapping of sterilizing breakpoints of inversions and deletions of the *D. melanogaster Y* chromosome does not give results different from those based on *T(X;Y)*s and *T(Y;A)*s (Gatti and Pimpinelli 1983). In addition, it has been shown in *D. hydei* that very small segments of the *Y* chromosome, translocated to the *X* chromosomal euchromatin, contain functional fertility genes. For example, *T(X;Y)340/10* contains only the distal-most portion of the long arm, forming a normally shaped loop pair *Threads* (Hess 1965b). This fragment represents approximately 10% of the *Y* chromosome, but it is able to complement mutations of the associated fertility gene *A* (Hackstein et al. 1982). Moreover, direct measurements in Miller spreads of transcribed chromatin of primary spermatocytes of *D. hydei* (Meyer and Hennig 1974; Glätzer and Meyer 1981; Grond et al. 1983; de Loos et al. 1984) and *D. melanogaster* (Glätzer 1980) also imply a large size for the loop-forming genes (also see section 5).

Since so many investigators arrived at similar conclusions, it seems likely that all genes on the *Y* chromosome that are mutable to complete male sterility have been identified. It cannot be excluded however, that there are additional fertility

genes that escaped detection because they only reduce fertility when mutated or deleted. *Y* chromosomal mutations that do not completely sterilize males would be difficult to detect, and in fact, have never been looked for. It is unlikely that there are *Y* chromosomal fertility genes that escaped detection because they have complementing alleles in the *X*-chromosomal heterochromatin of the *T(X;Y)s* used in the complementation tests (Kennison 1983).

Although the comparison of the mutation frequencies between genes from a heterochromatic chromosome and genes in located in the euchromatin may be misleading, it is clear that the six *Y* chromosomal fertility genes have relatively high mutation frequencies. The *X* chromosome, for comparison, carries an estima-

Fig. 1 A,B (facing page). The *Y* chromosomes of *D. hydei* and *D. melanogaster*, showing (**A**) the location of fertility genes and their correlation to the lampbrush loop pairs, and (**B**) lampbrush loop morphology in primary spermatocyte nuclei. In **A**, schematic representations of the (pro)metaphase *Y* chromosomes of both species are shown, as they appear after sequential staining with quinacrine, Hoechst 33258 and N-banding (Bonaccorsi et al. 1981; Gatti and Pimpinelli 1983). This permits the subdivision of the *Y* chromosome in 12 regions in *D. hydei*, and in 25 regions in *D. melanogaster*. Filled segments indicate bright fluorescence, grey segments indicate intermediately bright fluorescence, and open segments indicate no fluorescence. In *D. hydei*, the fertility genes are named *A*, *B*, *C*, *N*, *O*, *P* and *Q*. Abbreviations for lampbrush loop pairs are *Th*: *Threads*, *Ps*: *Pseudonucleolus*, *Tr*: *Tubular ribbons*, *Cl*: *Clubs* and *Ns*: *Nooses*. In *D. melanogaster*, the fertility genes are named *kl-5*, *kl-3*, *kl-2*, *kl-1*, *ks-1* and *ks-2*. Lampbrush loop pairs are *A*, *B* and *C*. The large *C* below each *Y* chromosome indicates the position of the centromere. In addition to the fertility genes, the *Y* chromosomes of both species carry other genetic functions. Multiple repeating subunits, each of them containing a gene for 28S, 18S, 5.8S and 2S ribosomal RNA, reside in the nucleolus organizers (*NO*) (reviewed by Ritossa 1976). There are two nucleolus organizers on the *Y* chromosome of *D. hydei*, and one on the *Y* chromosome of *D. melanogaster*. Other genes on the *Y* chromosome of *D. melanogaster* are the *Suppressor-of-Stellate* (*Su(Ste)*) gene, the position of which coincides with a gene required for male meiosis, and the *ABO* genes, which are responsible for the interaction with the *abnormal-oocyte* gene on chromosome *2*. The scale bar corresponds to approximately 10 megabase-pairs. **B** Schematic representation of *Y* chromosomal lampbrush loop pairs in primary spermatocyte nuclei of wild-type *D. hydei* and *D. melanogaster* males, as they appear in phase contrast microscopy of testis squashes. Abbreviations of lampbrush loop pairs are as in **A**, with, in the case of *D. hydei*, the addition of Th^c: compact part of *Threads*, Th^d: distal part of *Threads*, *Co*: *Cones*. The latter refer to the small projections of the *Pseudonucleolus*, which do not correspond to a separate loop pair since they cannot be correlated with a separate complementation group (Hackstein et al. 1982; Hackstein 1987). The lampbrush loops unfold from the nucleolus (*Nu*). As shown by Hennig (1967), the DNA axis of the long arm of the *Y* chromosome does not return to the nucleolus between two adjacent loop pairs. This determines the topological arrangement of the loop pairs within the nucleus. The pear-shaped Stage II primary spermatocyte nucleus of *D. hydei* has a length of about 40 µm and a diameter of about 35 µm, the round primary spermatocyte nucleus of *D. melanogaster* has a diameter of about 30 µm. Part **A** of this figure was based on Fig. 2 of Hennig (1985) and Fig. 14 of Hackstein (1987) (*D. hydei*) and on Fig. 1 of Gatti and Pimpinelli (1992) (*D. melanogaster*). Part **B** was based on on drawings of Hennig (1967) (*D. hydei*), and on an unpublished drawing provided by J.H.P. Hackstein (*D. melanogaster*)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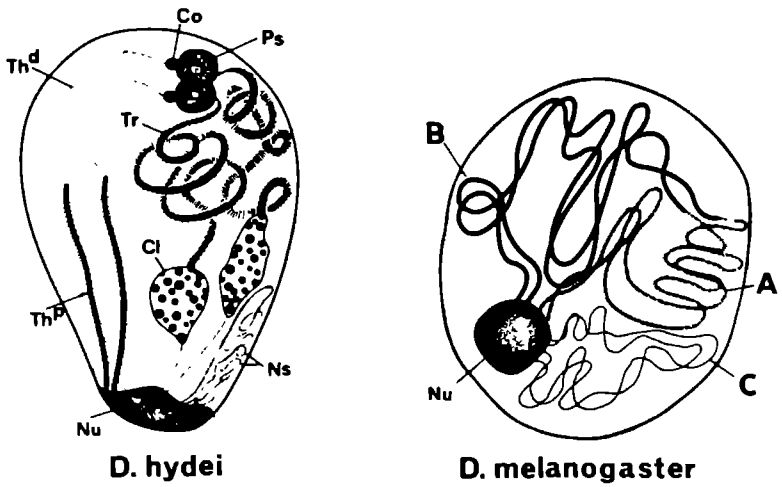
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ted number of 100-150 genes that can be mutated to male sterility (Lindsley and Lifschytz 1972), but the induction of male-sterile mutations by EMS or γ-rays generates about twice as much male-sterile mutations on the *Y* chromosome than on the *X* chromosome (Kennison 1983).

The high mutability of the fertility genes has been attributed to their large physical sizes (Gatti and Pimpinelli 1983). The mutation frequency per base pair is comparable with that of large (protein coding) genes on other chromosomes (Hackstein et al. 1991), such as the genes in the *bithorax*-complex of *D. melanogaster* (Tjiong et al. 1985). However, this issue is not resolved. Gene *kl-1*, which does not form a (visible) lampbrush loop pair, is not less sensitive than the loop-forming genes *kl-5*, *kl-3* or *ks-1* to mutational inactivation by either X-rays or by EMS (Gatti and Pimpinelli 1992). The initial claim for a large size of *kl-1* by Gatti and Pimpinelli (1983) is now considered to be incorrect by these authors. Another unresolved issue is the question whether the fertility genes contain mutational hotspots (Hennig 1993).

2.3 Fertility genes of *D. hydei*

Similar studies on the *Y* chromosomal fertility genes have been carried out in *D. hydei*. *Y* chromosomes of this species carrying visible markers are not available, but the lampbrush loops in primary spermatocyte nuclei serve as landmarks for the presence of certain segments of the *Y* chromosome (Meyer 1963; Hess and Meyer 1963a,b; Hess 1965a,b; also see section 3.2). The cytogenetic analysis of 125 *X-Y* translocations, 26 deficiencies of the *Y* chromosome, and 136 EMS and X-ray-induced male-sterile mutations on cytologically normal *Y* chromosomes, allowed Leoncini (1977) and Hackstein et al. (1982) to show that there are at least seven complementation groups on the *Y* chromosome. Six groups were mapped on the long arm, and one on the short arm. In the initial report 16 complementation groups were identified (named *A*, *B*, *C*, etc., up to group *Q*), but the validity of nine groups was uncertain (Hackstein et al. 1982; Hackstein 1987).

Each of the seven well-defined complementation groups was identified on the basis of multiple, independently induced, noncomplementing sterile mutations. Five were associated with one of the five lampbrush loop pairs, mapped by Hess (1965b). For example, group *A*, the most distal complementation group on the long arm, was found to be associated with the loop pair *Threads*. It was defined by 23 noncomplementing EMS-induced mutations and by 10 different deletions.

Also in *D. hydei* it was possible to use chromosome-banding techniques (Bonaccorsi et al. 1981) to map the fertility genes at defined positions on the metaphase *Y* chromosome (Hennig 1985; Hackstein 1987; also see Fig. 1). Together with the high mutation frequency (more than 5% of the mutagenized *Y* chromosomes), the distribution of the breakpoints of *X-Y* translocations indicated that the genes have physical dimensions in the megabase-range, confirming earlier measurements of loop length in the light microscope (Hess and Meyer 1968; Hennig et al. 1974b), and in Miller spreads of transcribed fertility gene chromatin (Glätzer and Meyer 1981; Grond et al. 1983; de Loos et al. 1984; also see section 5.2).

To summarize, both in number and in size, the fertility genes on the *Y* chromo-

some of *D. hydei* are quite comparable to those of *D. melanogaster*. Similar to *D. melanogaster*, it also appeared in *D. hydei* that large regions of the *Y* chromosome are devoid of fertility genes (Fig. 1).

2.4 Other *Drosophila* species

In the genus *Drosophila*, 8 subgenera, with a total of 613 species, were described by Patterson and Stone in 1952. In 1981, Wheeler listed 15 subgenera, with a total of 1467 species. In 1989, Ashburner listed 1677 species in these 15 subgenera, and it is possible that today, the number of known species has increased even further. However, the number and location of the *Y* chromosomal fertility genes have only been determined in one particular wild-type strain (Oregon-R) of *D. melanogaster* (Gatti and Pimpinelli 1983) and in one wild-type strain (Tübingen) of *D. hydei* (Hess 1965b; Leoncini 1977; Hackstein et al. 1982). These two species belong to the subgenera *Sophophora* and *Drosophila* of the genus *Drosophila*, repectively. These are the two largest subgenera, accounting for 280 and 825 species, respectively (Ashburner 1989), and they are thought to reflect an ancient subdivision that occurred appoximately 30 million years ago (Grimaldi 1990). As it is unclear to which extent the wild-type stocks used are representative for the species, and to which extent the two species from the two largest subgenera are representative for the entire genus, it is worthwhile to investigate the generatility of the conclusions from the previous sections.

Species with polymorphisms in the cytological appearance of the *Y* chromosome are listed in Table 1. In several species more than two different types of *Y* chromosomes can be distinghuished between different wild-type strains, including *D. melanogaster* (Halfer 1981). All these different types must carry the normal number of fertility genes, and therefore the polymorphisms affect only those regions of the *Y* chromosome that are dispensible for male fertility (see Fig. 1). Consistently, naturally occurring *Y* chromosomes of *D. melanogaster* exhibit no polymorphisms in male fertility nor in male fitness, but only if tested against the same genetic background (Williamson 1976; Clark 1990). Thus, in the case of *D. melanogaster*, the Oregon-R wild-type *Y* chromosome seems to be sufficiently representative.

It seems that in most *Drosophila* species, the *Y* chromosome carries fertility genes, as only eight species are known with fertile *X0* males. These species occur in three subgenera (Table 2). The *X0* males were either collected directly from nature, or generated in the laboratory as patroclinous sons from females that were subjected to a light X-ray treatment to enhance primary nondisjunction of the *X* chromosome. Three species are obligate *X0* species, *i.e.* *XY* males have never been found. In the other five species, fertile males are either *XY* or *X0*. In *D. affinis*, the *Y* chromosome does not display an all-or-none effect on male fertility, but it only provides a quantitative advantage in male fitness (Voelker and Kojima 1971). It must be assumed that in species with fertile *X0* males, genes on other chromosomes have evolved the ability to carry out the functions of the *Y* chromosomal fertility genes, or that these genes have been translocated to other chromosomes. In section 3.4, evidence is discussed that the fertile *X0* males also have lampbrush loop pairs (also see Hess 1980).

Table 1. *Drosophila* species with polymorphisms of the *Y* chromosome

subgenus	species group	species	reference
Sophophora	*melanogaster*	*D. melanogaster*	Halfer 1981
		D. simulans	Heitz 1933
	obscura	*D. pseudoobscura*	Dobzhansky 1935
		D. persimilis	Dobzhansky 1935
		D. affinis[2]	Miller and Stone 1962
		D. athabasca	Miller and Roy 1964
		D. algonquin	Miller and Stone 1962
		D. azteca	Miller and Roy 1964
		D. tolteca	Miller and Stone 1962
Drosophila	*repleta*	*D. serido*	Baimai et al. 1983

Notes:
[1] polymorphisms in size and shape as seen in orcein or quinacrine-stained mitotic metaphase preparations from spermatogonia or larval brain ganglia
[2] males lacking the *Y* chromosome are fertile in this species (see Table 2)

Table 2. *Drosophila* species with fertile *XO* males

subgenus	species group	species	male fertility *XO*	male fertility *XY*	references
Sophophora	*melanogaster*	*D. auraria*	+	+	1
	obscura	*D. affinis*[3]	+	+	1, 2
	obscura	*D. narragansett*	+	+	1
Drosophila	*annulimana*	*D. annulimana*	+	-	1
Hirtodrosophila	*longala*	*D. longala*	+	-	1, 3
	cinerea	*D. orbospiracula*	+	-	1, 3
	thoracis	*D. thoracis*	+	?	4
	?[4]	*D. pictiventris*	+	?	4

Notes:
[1] (+) fertile; (-) *XY* males not known to exist; (?) *XY* males exist, but it is unknown whether they are fertile
[2] References: (1) Voelker and Kojima 1971; (2) Miller and L. Stone 1962; (3) Patterson and W.S. Stone 1952; (4) Ashburner 1989
[3] The fertile *XO* males of this species have a lower fitness than *XY* males, as tested in population cages in the laboratory, see reference (1)
[4] Species group could not be traced

Thus, it seems reasonable to assume that almost all *Drosophila* species carry fertility genes on the *Y* chromosome (at least some of which form lampbrush loop pairs, see section 3.4). Only in some exceptional cases the genes have been translocated to other chromosomes. The functions of the *Y* chromosomal fertility genes must have been highly conserved during the evolution of the genus, since they have been subjected to a continuous and ruthless selection in every generation:

any mutation that affects fertility gene function reduces the fertility of the male carrying that mutation, or even renders such a male completely sterile. Therefore, it can be expected that in species as distantly related as *D. melanogaster* and *D. hydei*, the *Y* chromosomal fertility genes perform similar functions.

3 THE RELATIONSHIP BETWEEN FERTILITY GENES AND LAMPBRUSH LOOPS

3.1 The discovery of the *Y* chromosomal lampbrush loop pairs

When the *Y* chromosomal lampbrush loops were discovered in 1961, the existence of fertility genes on the *Y* chromosome of *D. melanogaster* had been known for decades. In that year, Meyer, Hess and Beermann described "phasenspezifische Funktionsstrukturen" in the nuclei of primary spermatocytes of *D. melanogaster*. Since these structures were not observed in the sterile males without the *Y* chromosome, and since some structures were missing in males lacking either the long or the short arm, it was concluded that the structures must represent the activity of genes on this chromosome. Both at the level of the light microscope and the electron microscope, the structures appeared highly similar to those formed by the lampbrush chromosomes (Rückert 1892) in the diplotene stage of amphibian oogenesis (see Callan 1986 for review). Moreover, in both systems, the structures were present in cells of the germ line, and only during meiotic prophase, when the DNA content of the cell is 4C. Around that time, the basic structure of the amphibian lampbrush chromosomes was understood (Callan 1986, 1987). Each lampbrush chromosome has an axis formed by two double-stranded DNA molecules, which at several positions along the chromosome unfold to form paired loops. In each loop one of the two DNA molecules becomes available for transcription (Gall 1956; Callan and Mcgregor 1958; Mcgregor and Callan 1962). The intranuclear structures in primary spermatocytes of *D. melanogaster* were therefore identified as lampbrush loops (Meyer et al. 1961; Meyer 1963).

This conclusion initiated extensive work on the *Drosophila* lampbrush loops, and since then, such loops have been detected in the primary spermatocytes of almost all *D.* species investigated (Hess 1967b), but in no species did the loops display such a rich morphological detail as in three closely related species from the *hydei* subgroup of the *repleta* group: *D. hydei*, *D. neohydei* and *D. eohydei* (Meyer 1963; Hess and Meyer 1963a,b). Of these species, *D. hydei*, a cosmopolitan species, was easiest to breed in the laboratory, and it became the subject of a number of investigations that eventually permitted to establish the relation between the loops and the fertility genes.

3.2 Lampbrush loop pairs of *D. hydei*

There are five lampbrush loop pairs in *D. hydei*, although in the initial descriptions of the loops by Meyer (1963a) and Hess and Meyer (1963a,b) only the four large, conspicuous loops on the long arm are mentioned. The names of these

four loop pairs are based on their morphological appearance: *Threads*, *Pseudonucleolus*, *Tubular ribbons* and *Clubs* (see Fig. 1). Several observations indicate that their formation is dependent on the *Y* chromosome. The loops are absent in *X0* males and all loop pairs are present in duplicate in (fertile) males carrying two *Y* chromosomes, and in interspecific hybrids of *D. hydei* and *D. neohydei* (which can be crossed in either direction), the morphology of the loops corresponded to that of the species donating the *Y* chromosome (Hess and Meyer 1963a). Hess (1965a) described two *Y*-linked, fertile mutations with a structurally modified *Threads* loop pair. Thus, all these observations imply that loop-formation is controlled *in cis* by the *Y* chromosome.

By studying males carrying fragments of the *Y* chromosome in *X*-*Y* translocations, Hess (1965b) was able to identify a fifth loop pair, called *Nooses*, formed by the short arm, and, in addition, to establish the linear order of the four loop pairs formed by the long arm, with *Threads* and *Pseudonucleolus* in a distal cluster, and *Tubular ribbons* and *Clubs* in a proximal cluster (Fig. 1). This was a major contribution to the mapping of the loop-forming genes and to the establisment of the correlation between the loop pairs and fertility genes: Hess observed that the absence of any of the loop-forming sites invariably caused complete male sterility. He therefore suggested that the genes forming the loops might be identical to the fertility genes. Strong support for this assumption came from complementation tests between different fragments of the *Y* chromosome, translocated either to the *X* chromosome or to an autosome (Hess 1967, 1968, 1970). Only combinations with all five loop-forming sites were displayed fertility. In addition, around this time Hennig (1967) had shown that the loops are sites of RNA synthesis (also see section 5.1).

Direct and unequivocal evidence for the correspondence of the loop pairs to fertility genes came from the following observations. Leoncini (1977) showed that *Y*-linked male-sterile mutations were of three types: (i) without cytological modifications in the lampbrush loops, (ii) with one or more loops absent, and (iii) with structural modifications of either all loops or of one specific loop. Subsequently, Hackstein et al. (1982) demonstrated that male-sterile alleles of certain complementation groups were correlated with modifications of a specific loop pair. Lack of complementation in group *A* was related with modification of the *Threads*. Similarly, group *B* was related with the *Pseudonucleolus*, group *N* with the *Tubular ribbons*, group *O* with the *Clubs*, and group *Q* with the *Nooses*.

Three important conclusions can be made on the basis of these studies. The most important conclusion is that each lampbrush loop pair is correlated with one male fertility gene, representing one complementation group. Second, not all fertility genes on the *Y* form a lampbrush loop pair that is visible in the light microscope (they may, however, form a loop pair that is too small to be identified). Third, noncomplementing, sterilizing alleles of a single loop-forming gene either leave the loop intact, modify the loop or cause its complete absence (Leoncini 1977; Hackstein et al. 1982, 1991; Hackstein 1987). However, morphologically modified loop pairs can also be functional. Hess (1965a) described X-ray-induced fertile alleles forming a modified *Threads*. Although such alleles somewhat reduce fertility (Hackstein et al. 1982), and may even cause complete sterility against

certain genetic backgrounds (unpublished observations of W. Hennig), Hess (1980) found that a strain of *D. hydei* that was classified as "wild-type" by the *Drosophila* Stock Center in Austin, Texas, contained a *Y* chromosome forming a modified *Threads* loop pair. This observation suggests that morphologically modified, but functional, loop pairs may occur even in natural populations.

3.3 Lampbrush loop pairs of *D. melanogaster*

The relatively poor morphology of the *D. melanogaster* loops had discouraged investigations of the relationship between loop pairs and fertility genes for almost 25 years. Phase contrast microscopy allows nothing more than the visualization of the loop pairs, enabling, at best, the detection of the absence of the largest and most conspicuous loop pair (loop pair *B*, see for example Fig. 3 of Hackstein 1991). Staining with Giemsa, Coomassie brilliant blue or the fluorescent dyes Hoechst 33258 and dansyl chloride considerably improves the visualization of some loop pairs, but this seems to be unsuited for detecting subtle alterations of loop morphology (Bonaccorsi et al. 1988).

Therefore, the relationships between the fertility genes and these loop pairs could be established only by immunofluorescence studies based on antisera that recognize unidentified, but loop-specific antigens (Hulsebos et al. 1984; Melzer and Glätzer 1985; Bonaccorsi et al. 1988).

The monoclonal antibody *S5* was raised against nuclear proteins from an embryonic *D. melanogaster* cell line (Saumweber et al. 1980). It recognizes a protein of M_r= 70 000 that is associated with newly transcribed RNA (Risau et al. 1983). In *D. hydei* it reacts with epitopes on the *Threads* and *Tubular ribbons* (Glätzer 1984), in *D. melanogaster* with epitopes on two lampbrush loop pairs (Melzer and Glätzer 1985), that were named *A* and *C* by Bonaccorsi et al. (1988). The *S5*-staining pattern of loop pair *A* is modified in several sterilizing alleles of gene *kl-5*, whereas other sterilizing alleles display a normal staining pattern of the loop pair. Similarly, also the *S5*-staining patterns of loop pair *C* can be modified or normal, depending on the particular sterile allele of *ks-1* (Bonaccorsi et al. 1988).

The polyclonal antiserum *sph155* was raised against a major sperm protein of *D. hydei* of M_r= 155 000 (Hulsebos et al. 1983). It decorates the *Pseudonucleolus* loop pair in *D. hydei* and one loop pair in *D. melanogaster* (Hulsebos et al. 1984). This loop pair was named *B* and its staining pattern is modified in some, but not all, sterile alleles of fertility gene *kl-3* (Bonaccorsi et al. 1988).

Thus, loop pair *A* is correlated with fertility gene *kl-5*, loop pair *B* with gene *kl-3*, and loop pair *C* with gene *ks-1* (see Fig. 1). Gatti and Pimpinelli (1983) had earlier determined the locations of each of these genes by mapping the positions of sterilizing, noncomplementing breakpoints in the metaphase *Y* chromosome. Using the *sph155* and *S5* antisera, this analysis was repeated by Bonaccorsi et al. (1988) for delimitating the loop-forming regions (see Fig. 1). Breakpoints preventing unfolding of loop pair *A* were clustered in the proximal part of *kl-5*, defining an estimated size of 1300 kb for the loop. Sterilizing breakpoints in the distal two-thirds of the 4000 kb occupied by *kl-5* had no apparent effect on loop morpho-

logy. Similarly, only breakpoints located proximally in *ks-1* were shown to affect the unfolding of loop pair *C*, defining as the loop-forming region the most proximal 1300 kb within the 4000 kb *ks-1* gene. For loop pair *B*, all breakpoints affecting the function of *kl-3* also affected the unfolding the loop pair, and thus the loop-forming region is coextensive with the 4300 kb fertility gene (Gatti and Pimpinelli 1992). Thus, for two of the loop pairs of *D. melanogaster* it seems that the fertility gene is considerably larger than the loop-forming region. It is unknown whether this is also the case for any of the loop-forming genes of *D. hydei*.

3.4 Other *Drosophila* species

Only in *D. melanogaster* and *D. hydei* the relationship between the loop pairs and the fertility genes has been identified. Detailed descriptions of lampbrush loop cytology are available only for several other species that are closely related to either of these two species. By inference, it is assumed that in these species as well, each loop pair is associated with one fertility gene (see Hess 1980).

Meyer (1963) performed an electron microscopic study of the loop pairs of *D. simulans*, a sibling species of *D. melanogaster*. Remarkably, these two species, which can be hybridized, were found to display some conspicuous differences in the ultrastructure of the loops. Similarly, comparisons in primary spermatocyte cytology, both at the light and electron microscopic level, between *D. hydei*, its sibling species *D. neohydei* (Meyer 1963; Hess and Meyer 1963a; I. Hennig 1978, 1982), and the somewhat more distantly related species *D. eohydei* (I. Hennig 1978) revealed that these species have a similar number of loop pairs, with similar, but species-specific morphology. It has even been shown that a fragment of the *D. neohydei Y* chromosome, translocated to the *D. hydei X* chromosome, can complement a deficiency of fertility gene *A* (forming the *Threads* loop pair) of *D. hydei* (I. Hennig 1982). Thus, although differing in morphology, at least one of the loop-forming fertility genes is functionally similar between these two species.

Beyond the species mentioned above, a description of loop morphology at the electron microscopic level is available only for *D. repleta* (Meyer 1963). For some 55 other species, representing 5 subgenera, the lampbrush loop pairs, as they appear in phase contrast optics, have been described by Hess (1967b), Hess and Meyer (1968), and Hess (1980). These studies show that loop morphology is highly specific for each species, and can even be used reliably for species identification (Meyer 1965). Conspicuously large lampbrush loop pairs were found in some, but not in all, species of the *repleta* group, which contains 72 species (Wasserman 1982). Especially *D. hydei*, *D. neohydei*, *D. eohydei*, *D. nigrohydei* and *D. bifurca* (all from the *hydei* subgroup) and *D. fulvimacula* (from the *melanopalpa* subgroup) displayed such loop pairs. In no species however, are the primary spermatocyte nuclei "empty": in all *Drosophila* species studied the nuclei display structures that are either granular, tube-like or thread-like. Also in some of the exceptional species with fertile *XO* males listed in Table 2, lampbrush loops have been observed in primary spermatocyte nuclei by Hess (1980), although it is not specified in which of these species. Therefore, it seems that in species with fertile *XO* males, the loop-forming fertility genes have been translocated to other chromosomes.

Combining the data described in this section with those from section 2.4, it can be concluded that the genes forming the morphologically dissimilar lampbrush loop structures are likely to perform similar, evolutionary conserved, functions in the different species. These functions have been studied in detail only in *D. melanogaster* and in *D. hydei*, as will be discussed below.

4 THE FUNCTIONS OF THE FERTILITY GENES DURING SPERMATOGENESIS

To gain insight into the biological functions of the loop-forming fertility genes, several investigators have studied spermatogenesis in males that lack the entire *Y* chromosome or they studied males carrying deletions or mutations of specific *Y* chromosomal fertility genes. Following a general description of spermatogenesis and its genetic control, the results of these studies will be summarized and

4.1 General aspects of spermatogenesis

Spermatogenesis is the development of the male germ cell within the male gonad. Fig. 2 gives a schematic overview of the morphogenetic changes of the male germ cell as they are seen in the light microscope. The male germ cells are descendants of the pole cells, the first cells to be formed in the *Drosophila* embryo, and colonize the developing male gonad during embryogenesis (Campos-Ortega and Hartenstein 1985; Technau and Campos-Ortega 1986). In adult males, the testis is a steady state system in which all the successive stages of spermatogenesis are present (Lindsley and Tokuyasu 1980). All stages are well defined, both at the level of the light microscope and the electron microscope. Concise accounts of spermatogenesis in *D. melanogaster* have been given by Cooper (1950), Hess and Meyer (1968), Kiefer (1971), and Tates (1971). Details at the ultrastructural level can be found in the different papers of Tokuyasu and associates, which are reviewed by Lindsley and Tokuyasu (1980). Spermatogenesis in *D. hydei* has been described in detail by Hess and Meyer (1968), Grond (1984), and Hennig and Kremer (1990), and for several other species, short descriptions have been given by Jamieson (1987).

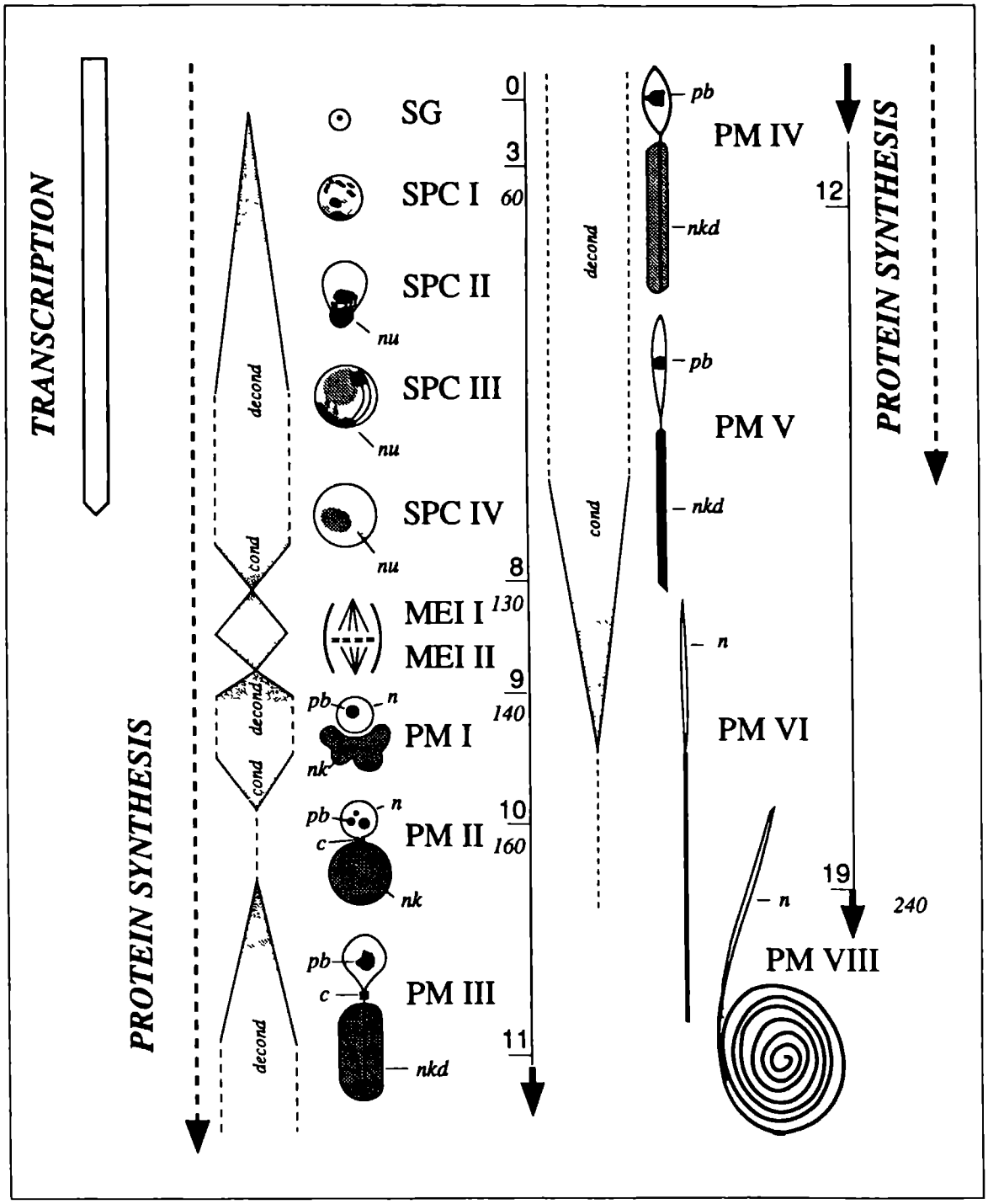
Briefly, spermatogenesis starts at the tip of the coiled testis tube, where germ line stem cells divide to generate spermatogonia, and somatic cyst progenitor cells devide to produce cyst cells. A single spermatogonial cell is surrounded by two somatic cyst cells (see Gönczy et al. 1992 for details). Within these cyst cells, the spermatogonia proliferate by mitotic divisions into cysts of 8 primary spermatocytes in the case of *D. hydei*, and 16 in the case of *D. melanogaster*. Until their individualization, the germ cells remain within the cysts, and they are connected by cytoplasmic bridges. All cells of a cyst develop in synchrony.

The primary spermatocyte stage in *D. hydei* was divided in several stages by Hennig (1967), based on cytological criteria (also see section 5.1). Stage 0 represents interphase, including the S-phase, Stages I to IV represent meiotic prophase I (Kremer et al. 1986). The customary terminology describing the different stages

of meiotic prophase (leptotene, zygotene, pachytene, diplotene and diakinesis) does not apply here. Due to the somatic pairing of the chromosomes, which is typical for Dipterans (White 1973), the chromosomes are already paired at the beginning of meiotic prophase. In addition, the chromosomes remain in a dispersed state throughout the entire prophase, with a very fast condensation occurring only shortly before metaphase (Cooper 1950; Kremer et al. 1986), and chiasmata are not formed during male meiosis in the higher Dipterans (Gehtmann 1988). Meiotic prophase I is the longest of the different stages of spermatogenesis, and in *D. hydei* it lasts for about half the time required to complete the entire process. This stage is characterized by an increase in cell volume and by an intensive transcription of the entire genome, including the *Y* chromosome, which unfolds to form the lampbrush loop pairs (see section 5.1).

At the end of the primary spermatocyte stage the loops disappear, the cell membrane disintegrates, and the meiotic spindle is formed. The two meiotic divisions occur and each cyst now contains 32 (*D. hydei*) or 64 (*D. melanogaster*) spermatids. These start a program of radical morphogenetic changes, affecting almost every cellular organelle. Each young spermatid consists of a round (haploid) nucleus (containing a conspicuous structure called protein body), an acroblast, an irregularly shaped nebenkern and the centriolar adjunct, which connects nucleus and nebenkern. The nebenkern is a fusion product of the mitochondria. The spermatids elongate by a factor of more than 1000 in *D. hydei*, and more than 150 in *D. melanogaster* during the postmeiotic stages: the nuclei elongate, the axoneme extends from the centriole, and the nebenkern derivatives elongate together with the growing axoneme. During advanced elongation stages, the nebenkern derivatives become filled with an electron-dense material, with a regular substructure, the so-called paracrystalline material (see for details Meyer 1964; Miedema 1994). When

Fig. 2 (facing page). Schematic representation of spermatogenesis in *D. hydei* (after Hackstein 1991). Spermatogonial cells (*SG*) undergo three mitotic division cycles to produce cysts of eigth primary spermatocytes (*SPC*). Five different stages of primary spermatocyte development have been defined by Hennig (1967), named *SPC 0* (not shown) and *SPC I–IV*, during which the *Y*-chromosomal lampbrush loop pairs unfold from the nucleolus (*nu*). They are maximally extended during *SPC II–III*, and are decomposed during *SPC IV*, shortly before meiosis (*MEI*). The short secondary spermatocyte stage between meiosis I and II is not shown. During the postmeiotic (*PM I–VIII*) stages, the nucleus (*n*), the protein body (*pb*) within the nucleus, and the nebenkern (*nk*) undergo characteristic changes in shape. The nucleus elongates considerably. The nebenkern, which is formed during meiosis from the fused mitochondria, is irregularly shaped during PM I, becomes round during PM II, it is unequally split in two parts during PM III and then elongates together with the growing axoneme, which extends from the centriolar adjunct (*c*). After elongation, the spermatids individualize, they coil and attain motility. During spermatogenesis of *D. hydei*, the chromatin is subjected to several cycles of condensation/decondensation, indicated in the column marked *cond./decond.* (Kremer et al. 1986). Arrows, marked *transcription* and *protein synthesis*, indicate the periods for RNA and protein synthesis, respectively (Hennig 1967). The timescale for spermatogenesis in *D. hydei* is indicated with *large* numerals (representing *days*), and for *D. melanogaster* with smaller, *italic* numericals (representing *hours*)


TRANSCRIPTION
PROTEIN SYNTHESIS
PROTEIN SYNTHESIS
SG
SPC I
SPC II
SPC III
SPC IV
MEI I
MEI II
PM I
PM II
PM III
PM IV
PM V
PM VI
PM VIII
decond
cond
nu
pb
n
nk
c
nkd
0
3
60
8
130
9
140
10
160
11
12
19
240

elongation has been completed, the spermatids individualize (*i.e.* each spermatid becomes surrounded by its own membrane), while their heads remain anchored in the head cyst cell. Simultaneously, they coil and they are transferred to the testicular duct. At the end of their passage through the duct, they become individually motile (see Grond 1984 for details).

It has been shown by Grond (1984) and Kremer et al. (1986) that during spermatogenesis in *D. hydei* the genome becomes condensed and decondensed several times, before it is finally packaged into the sperm head. Such condensation/decondensation cycles, which may reflect a reprogramming of the male genome (Hennig 1985, 1987b), occur most likely in *D. melanogaster* as well, as there is evidence from cytochemical studies for a substitution of lysine-rich by arginine-rich chromosomal proteins (Das et al. 1964; Hauschteck-Jungen and Hartl 1982).

Spermatogenesis is very conserved throughout the animal kingdom (see for example Hennig 1992), and spermatogenesis in *D. melanogaster* differs from that in *D. hydei* only in some minor aspects (Hennig and Kremer 1990). The most conspicuous differences, in addition to the size of the lampbrush loops and the number of gonial divisions (see above) concern the length of the mature sperm cells, which is 1.8 mm in *D. melanogaster* (Cooper 1950) and more than 12 mm in *D. hydei* (Grond 1984; Hennig and Kremer 1990), and the total time required for spermatogenesis, which is less in *D. melanogaster* (see Fig. 2). In both species, however, the timing of the different stages is tightly controlled (Hennig 1967; Leoncini 1977; Lindsley and Tokuyasu 1980).

4.2 The function of the *Y* chromosome during male germ cell development

The genetic basis of germ cell development involves three basic determinative events (Hennig and Kremer 1990): (i) the establishment of undifferentiated cells as germ cells during early embryogenesis, (ii) the determination of the sex of the germ line cells, (iii) the differentiation of the germ line cells, *i.e.* the decision to perform either oogenesis or spermatogenesis. These events are followed by the execution of either developmental process. As discussed below, the *Y* chromosome is required only during the differentation of the male germ cells.

ad (i): The *Y* chromosome does not play a role in the formation of the pole cells during early embryogenesis. *XO* embryos form pole cells, which become included in a normal testis, but *XO* pole cells do not fully develop to motile sperm (see section 4.5). Pole cell formation is under the control of a group of cooperating maternal-effect genes, leading to the localized deposition of pole cell determinants at the posterior end of the egg (reviewed by Mahowald 1992).

ad (ii): The *Y* chromosome has no role in the sex determination of the germ line cells. Steinmann-Zwicky et al. (1989) transplanted chromosomally female (*XX*) pole cells into agametic, male (*XY*) host embryos (such embryos, that lack pole cells of their own, are derived from mothers homozygous for the maternal-effect mutation *oskar*^{301}). The transplanted *XX* pole cells assumed a male sex, and entered the male pathway of germ cell development (Steinmann-Zwicky et al. 1989). Thus, the female sex-determining signal (an *X*:autosome ratio of 1) is overruled when *XX*

pole cells become included within a testicular environment, indicating that there are inductive influences between the somatic cells of the testis and the germ line cells.

ad (iii): The *Y* chromosome is not required for entering the male pathway of germ cell development. The *XX* pole cells transplanted by Steinmann-Zwicky et al. (1989) developed into primary spermatocytes. In addition, also the inductive influences from the somatic environment of the testis (or from the somatic paragonial glands) do not require the *Y* chromosome: Marsh and Wieschaus (1978) transplanted pole cells from *XY* donor embryos into *XO* host embryos. Normally, *XO* embryos would develop into sterile adults with normal testis and normal paragonial glands. However, the embryos which had received *XY* pole cells developed into fertile adults that were able to produce viable, fertile offspring. Most importantly, this experiment also implies that spermatogenesis is autonomously performed by the germ cell, a conclusion that is consistent with the fate mapping experiments of Nissani et al. (1978). Using *XY/XO* mosaic males, these authors mapped the location of *Y* chromosome function in the germ cells. Thus, the sterility of males carrying mutations in *Y* chromosomal fertility genes is entirely attributable to defects in germ cell function.

In summary, the genetic function of the *Y* chromosomal fertility genes is required only after the male germ cells have started the execution of the program of spermatogenesis. Before dealing with his function, the progress that has been made in the genetic dissection of spermatogenesis in *D. melanogaster* and *D. hydei* will be briefly reviewed. The similarity of spermatogenesis in these two species is not only obvious from a comparison of the morphogenetic changes of the germ cells. Also the phenotypes caused by several male-sterile mutations (Kiefer 1973; Lifschytz 1987; Hackstein et al. 1990; Hackstein 1987, 1991; see section 4.4) imply that the genetic control of spermatogenesis, and, by inference, its underlying molecular mechanisms, are very similar.

4.3 Conserved molecular mechanisms in the genetic control of spermatogenesis

The execution of postmeiotic sperm morphogenesis in *Drosophila* is performed without concomitant transcription, whereas translation occurs until the individualization stage, both in *D. melanogaster* (Olivieri and Olivieri 1965; Gould-Somero and Holland 1974), and in *D. hydei* (Hennig 1967). The only exception to this general rule is the 93D heat shock gene of *D. melanogaster*, which does not encode a protein, but intranuclear RNAs of unknown function (Pardue et al. 1987). This gene is expressed after meiosis (unpublished observations of M.L. Pardue et al., cited by Erickson 1990). The general absence of haploid gene expression during *Drosophila* spermatogenesis contrasts with the situation in mammals, where many cases of haploid gene expression have been documented (Handel 1987; Willison and Ashworth 1987; Erickson 1990).

In fact, postmeiotic spermatid development in *Drosophila* is normally performed without the *X* or the *Y* chromosome in half of all the germ cells. In *D. melanogaster* it may also be performed without major portions of the large autosomes (McCloskey 1966), and it can even be performed with only the tiny fourth chromo-

some present: spermatids that lack either *X* or *Y* and both the large autosomes develop into motile, functional sperm (Lindsley and Grell 1969). A limitation to this statement is that it cannot be excluded that before individualization, some gene products are transferred from cell to cell, since the elongating spermatids develop in cysts and are connected by cytoplasmic bridges.

Because of the general absence of haploid gene expression, the morphogenesis of the sperm cell must be regulated by other mechanisms than differential gene expression. Little is known about these mechanisms, but the available data show that, like any other differentiation process, *Drosophila* spermatogenesis involves the controlled utilization of mRNAs.

In both *D. melanogaster* and *D. hydei*, some mRNAs that encode components of the sperm cell are translated before meiosis, whereas others are stably stored for many days until their recruitment by the translational apparatus. The β_2-tubulin gene belongs to the first type, since the earliest effects of mutations in this gene occur during meiosis (Kemphues et al. 1979, 1980, 1982). The coding sequences of the gene as well as sequences for its transcriptional control are conserved between the two species (Michiels et al. 1987), and both mRNA and protein are detectable as early as during the primary spermatocyte stage (Michiels et al. 1989; Kaltschmidt et al. 1991).

The *Mst(3)CGP* tail protein gene family of *D. melanogaster* belongs to the second type. These proteins are thought to form the electron-dense material in the accessory microtubules of the axoneme, which appears 5 days after meiosis (Kuhn et al. 1988; M. Schäfer et al. 1990, 1993). *Cis*-acting sequence motifs that prevent precocious translation during early postmeiotic stages have been identified in several mRNAs encoding proteins of the *Mst(3)CGP* family in *D. melanogaster* (M. Schäfer et al. 1990;1993). At least three members of the gene family have been identified in *D. hydei*, and at least one of them has identical *cis*-acting translational control sequences (unpublished observations of H. Bünemann, cited by M. Schäfer et al. 1993). These signals, however, are not present in transcripts of all genes encoding sperm tail proteins in *D. hydei*: they are absent in the *Dhmst101(1)* gene, encoding a different testis-specific, axoneme-associated protein, that is absent in *D. melanogaster* (Neesen et al. 1993).

In addition to the controlled utilization of pre-synthetized mRNAs, it is likely that also self-assembly is a major factor controlling postmeiotic spermatid development (Hennig 1987b). Several major proteins of the mature sperm cell have a repetitious substructure, and are likely to be built up by self-assembly processes, such as, for example, the paracrystalline material within the nebenkern derivatives (Meyer 1964; Miedema 1994). The polymerization of α- and β_2-tubulin into the microtubules of the axoneme occurs by self-assembly, and possibly the incorporation of the various microtubule-associated proteins, such as the dyneins, as well (reviewed by L. Amos and W. Amos 1991). The tubulins and the dyneins are highly conserved proteins of a conserved axonemal structure.

These few examples illustrate that control of gene expression during *Drosophila* spermatogenesis is a highly conserved phenomenon, as is also obvious from the analysis of male-sterile mutations described in the next sections. Thus, the lampbrush loop-forming fertility genes, which are likely to perform conserved

functions in all *Drosophila* species, are part of a conserved system of regulatory relationships between specific genes. Remarkably, the functions of the loop-forming fertility genes do not require conservation of loop morphology.

4.4. The genetic control of spermatogenesis: mutations causing male sterility

The genetic dissection of spermatogenesis has been attempted systematically only in *D. melanogaster*, and, together with the concomitant construction of balancer chromosomes (Hackstein et al. 1992), also in *D. hydei*. The study of mutations causing male sterility, including mutations of the loop-forming fertility genes, is difficult for several reasons. As discussed by Hennig (1987b), Lifschytz (1987), Lindsley and Tokuyasu (1980), Hennig and Kremer (1990) and Hackstein (1991), the phenotypic defects of male-sterile mutations may not always directly point at the function of the affected gene. In many mutants, the spermatids develop to completion but they remain immotile and subsequently, they degenerate. Even in wild-type males of *D. melanogaster*, spermatids may undergo a process of degeneration (Bairati 1967; Meyer 1969; Koopmans-Frankel et al. 1971; Kiefer 1973). Other male-sterile mutations also affect somatic tissues. These two categories of genes are most likely responsible for general metabolic processes. Spermatogenesis seems to be particular sensitive to such disturbances (Lindsley and Tokuyasu 1980; Hackstein 1987), since, for unknown reasons, 50% of all genes appear to be expressed in the testis (Bownes 1990). Lindsley and Tokuyasu (1980) estimate that approximately 1250 genes can be mutated to male sterility. It is unlikely that all these genes are specifically required for executing the program of sperm morphogenesis, since, for example, the deposition of maternal information in the egg and its subsequent elaboration during embryogenesis is controlled by not more than 50 genes (Ingham 1988; St Johnston and Nüsslein-Volhard 1992).

Other categories of male-sterile mutations affect the mating behavior of the male flies, the copulatory organs, the growth of the testis or the somatic cells of the testis, or they affect the paragonial glands. It is however, possible to distinguish such mutations from those interfering only with sperm morphogenesis (Hackstein 1991; Castrillon et al. 1993).

Therefore, certainly not more than 20% of all male-sterile mutations have the male germ cell as the primary target. In general, such mutations can be classified according to their effect on the development of the male germ cell (Lifschytz 1987; Hackstein et al. 1990; Hackstein 1991; Castrillon et al. 1993). A minority of mutations cause a distinct stop at a certain stage of spermatogenesis, either interfering with mitotic proliferation, with spermaticyte growth, with entry into meiosis, or with meiosis itself.

Most mutations, including those in the *Y* chromosomal fertility genes (Meyer 1968; Kiefer 1973; Hardy et al. 1981; also see section 4.5), result in defects in post-meiotic spermatid differentiation (Hackstein 1991). Some of these mutations affect several or all components of the developing sperm cell synchronously, others specifically affect the development of only one particular component, such as the nebenkern, the nucleus, or the protein body. In such de-synchronizing mutants

the other components develop normally, even if the affected component is completely missing (Hackstein et al. 1990; Hackstein 1991; Castrillon et al. 1993). Some mutations even allow the formation of motile sperm which can be transferred to the female. In these rare cases (Hackstein et al. 1991) sterility is either the result of the inability of the sperm to fertilize or, if fertilization does occur, of paternally-induced embryonic lethality.

Thus, from a genetic analysis, spermatogenesis appears as "the coordinate execution of the individual developmental programs of the different components of the spermatozoon" (Hackstein et al. 1990). However, mutations can result in complex male-sterile phenotypes. Therefore, it may be difficult to correlate a phenotypic defect with the function of a mutated gene product. Even mutations in a structural sperm protein as β_2-tubulin are pleiotropic, causing defects in meiosis, nuclear morphology and axoneme formation (Kempues et al. 1979, 1982), suggesting at first sight, a regulatory role for the gene product. Thus, without the availability of molecular probes it may not possible to ascertain whether gene products specifically required for spermatogenesis have a structural or a regulatory role (or perhaps even both). In addition, there is also the danger of confusing specific developmental defects and non-specific degeneration of the spermatids, which even occurs in wild-type males. It is with these consideration in mind that the effects of mutations and deletions of the *Y* chromosomal fertility genes should be discussed.

4.5 Male-sterile phenotypes of the loop-forming fertility genes

4.5.1 *X0* males

The earliest differences between spermatogenesis in *XY* and *X0* males of *D. melanogaster* are visible at the primary spermatocyte stage, when the lampbrush loop pairs are absent in *X0* males (Meyer et al. 1961; Meyer 1968). Another difference concerns the the formation of crystals in the primary spermatocytes. In wild-type strains, such crystals are occasionally observed, and they can be induced by ligation of larvae (Meyer 1969), but in *X0* males, they are always found. Depending on the allelic state of the *X*-linked *Stellate* gene, the crystals in *X0* males are either needle-shaped or star-shaped (Meyer et al. 1961; Hardy 1980; Hardy et al. 1984; Livak 1984). *X0* males of *D. hydei* do not have crystals, also not at the level of the electron microscope (Hess and Meyer 1963a, 1968).

Also meiosis is different in *X0* males compared to *XY* males. Frequently, nondisjunction is observed, as shown by direct examination of anaphases in orcein-stained testis squashes of *X0* males (Lifschytz and Hareven 1977; Lifschytz and Meyer 1977), by the reduced number of spermatids in developing cysts (Kiefer 1966) and also by the different sizes of the round nuclei of the young spermatids immediately after meiosis (Hardy et al. 1984). As shown by Hardy (1975) and by Gonzalez et al. (1989), the volume of early spermatid nuclei is a sensitive and reliable indicator of defects in chromosome distribution during meiosis. The gene responsible for the meiotic defects in *X0* males has been mapped between *kl-2* and *kl-1* (Hardy et al. 1984). However, this gene is not a fertility gene, since in

combination with certain alleles of the *X*-linked *Stellate* gene, males lacking the gene are fertile. The *Y* chromosome of *D. hydei* may not carry such a gene, as meiosis in *XO* males of this species does not display the irregularities described above for meiosis in *XO* males of *D. melanogaster* (see below).

Althought meiosis is disturbed, spermatids are nevertheless formed, containing a nucleus, an axoneme and a nebenkern derivative. Both Meyer (1969) and Kiefer (1973) concluded that the sterility of *XO* males of *D. melanogaster* is caused by an aberrant differentiation of the spermatids, which do not reach the length of wild-type sperm (1.0–1.2 versus 1.8 mm). Light microscopical observations of J.H.P. Hackstein (personal communication) even indicate that spermatids of *XO* males can individualize, but never attain motility. At the ultrastructural level, defects in the structure of the axoneme may be detected, as well as irregularities in the arrangement of axoneme and nebenkern derivatives (Kiefer 1966, Meyer 1968), and from these investigations Meyer (1969) arrived at the following conclusion: "It seems that *XO* spermatids in *D. melanogaster* contain all the structural components of normal sperm, but fail to organize them properly". As discussed by Kiefer (1973), it is difficult to exclude that such defects are the result of spermatid degeneration. Nevertheless, also this author concluded that "all the necessary structural elements discernible at the level of the electron microscope appear to be present in *XO* spermatids". As shown by Hardy et al. (1981), the conclusions of Meyer (1969) and Kiefer (1973) were not entirely justified, as males lacking either fertility gene *kl-5* or *kl-3* have no outer dynein arms in the axonemal microtubules (see below).

With respect to *XO* males of *D. hydei*, the initial studies of Hess and Meyer (1963a, 1968) claimed that spermatogenesis is arrested during the primary spermatocyte stage. This is not correct, as discussed by Hennig et al. (1974a). At a breeding temperature of 24°C, meiosis is abnormal and there is almost no postmeiotic development. At lower temperatures (18°C), meiosis is normal (in contrast to *D. melanogaster*), with a regular segregation of the chromosomes, as is evident from the equal volumes of the round spermatid nuclei and from microspectrophotometry of Feulgen-stained spermatid nuclei, which do not reveal detectable deviations from a haploid DNA content (H. Zacharias, personal communication). Spermatids are formed, and in aged *XO* males a considerable elongation of the spermatids is observed, which, however, do not individualize. Although spermatid elongation is variable, the spermatids are not detectably abnormal at the level of the light microscope (unpublished observations of H. Kremer and W. Hennig, cited by Hennig 1990). Based on ultrastuctural studies, Meyer (1972) even concluded: "It appears that all structural components of sperm organelles are present in *Y* deficient males but fail to be organized in complexes of typical architecture."

What can be learned from the phenotypic analysis of spermatogenesis in *XO* males about the possible functions of the *Y* chromosomal fertility genes? A major conclusion is that the absence of the *Y* chromosome does not inhibit the basic morphogenesis of the sperm. Whereas it is not possible to conclude whether the fertility genes function at a regulatory or at a structural level, the studies of *XO* males do seem to indicate that, if there are structural genes on the *Y* chromosome, they do not encode abundant proteins of the sperm. Initial comparative analyses of testis proteins from *XO*, *XY* and *XYY* males of *D. melanogaster* by two-dimensional

polyacrylamide gel electrophoresis (PAGE) indeed failed to reveal quantitative and qualitative differences in protein content (Ingman-Baker and Candido 1980). This study was, however, critized by Goldstein et al. (1982), because testis proteins of a high molecular weight were not sufficienty resolved in the gel system. Goldstein et al. (1982) were able to show that several sperm proteins of a high molecular weight ($M_r > 200\,000$) are absent in *X0* males, and they concluded from these, and other, data that certain *Y* chromosomal fertility genes are protein coding genes (also see section 4.6). In *D. hydei*, quantitative differences in protein content between testis from *XY* and *X0* males have been demonstrated (Hulsebos et al. 1983), but these differences concern proteins that are not encoded on the *Y* chromosome.

4.5.2 Males carrying deletions or mutations of individual fertility genes

The early studies of the phenotypic defects caused by deletions and mutations of individual fertility genes, as identified by Brosseau (1960) on the *Y* chromosome of *D. melanogaster*, have been reviewed by Hess and Meyer (1968), Kiefer (1973) and Williamson (1976). Although the application of the electron microscope in most studies, the results were "disappointing" (Kiefer 1973), mainly because it was difficult to discriminate between specific development defects and nonspecific spermatid degeneration.

Nevertheless, in 1981, Hardy et al. succeeded in identifying the primary developmental defect of deletions of some, but not all, fertility genes. Deletions of either *kl-5* or *kl-3* had a specific defect: both caused the absence of the outer dynein arm of each of the nine peripheral microtubule doublets in the axoneme. Deletion of *kl-2* had almost the same phenotype as the deletion of the entire *Y*. Deletions of either *kl-1* or *ks-1* caused very similar, subtle defects such as the proper apposition of the spermatids within a cyst. Kiefer (1968,1969) had earlier reported that *kl-1*-deficient males form motile sperm that is transferred to the female, but that is incapable of fertilization. However, Hardy et al. (1981) could not confirm the observation of sperm motility in *kl-1*-deficient males. Deletion of *ks-2* prevented a normal arrangement of axoneme and nebenkern. Thus, similar to the situation in *X0* males, there was no major sperm organelle that could not be formed in the absence of a particular fertility gene, with the exception of the outer dynein arms of the axoneme.

The analysis of *D. hydei* males lacking individual *Y* chromosomal fertility genes gave results that were very comparable to those in *D. melanogaster* (Hess and Meyer 1968; Meyer 1968, 1969, 1972; Hackstein et al. 1982; 1991). Deletions of gene *Q*, forming the *Nooses* loop pair, caused a developmental arrest before individualization of the completely elongated spermatids. The deletion of any of the other individual loop-forming fertility genes caused an arrest after individualization, with immotility of the sperm as the only apparent defect at the level of the light microscope (Hackstein et al. 1991). The male-sterile mutation *ms(Y)Kl*^{ts} causes an absence of the electron dense structures in the central microtubule doublet of the sperm axoneme (Leoncini 1977), but it is unknown which complementation group is affected in this mutant (Hackstein et al. 1982). Deletions of only gene *A*,

forming the *Threads*, even permits some coiling of the individualized, but immotile spermatids. Of course it cannot be excluded that other defects occur which are recognized only at the level of the electron microscope. For example, deletions of either gene *A* or gene *N* (forming the *Tubular ribbons*) caused the absence of the outer dynein arms in the sperm axoneme (Kociok, cited by Hackstein et al. 1991). This observation strongly suggests that the functions of these two loop-forming fertility genes are similar to those of *kl-5* and *kl-3* of *D. melanogaster*, although loop morphology is entirely different between these two species.

A comparison of the phenotypes caused by deletions and mutations of the same fertility gene did not reveal differences in the severities of the effects on spermatid development (Leoncini 1977; Hackstein et al. 1982). In addition, a comparison of the phenotypic defects caused by the different male-sterile alleles of gene *A*, forming the *Threads*, and gene *C*, forming the *Pseudonucleolus*, did not reveal differences at the level of the light microscope. Irrespective of whether the corresponding loop pair was normally formed, modified, or not formed at all, the male-sterile phenotype was identical (Hackstein et al. 1991). Similarly, an unpublished investigation by R.W. Hardy of 42 sterile mutations of *kl-5*, *kl-3*, or both, revealed the absence of the outer dynein arms in all alleles, irrespective of whether the lampbrush loop pairs *A* and *B* were normally formed, modified, or absent (cited by Hackstein et al. 1991).

Thus, to summarize, deletions or mutations of individual fertility genes cause similar and relatively mild defects, which become evident only at the end of spermatid development. However, from these phenotypic defects alone, no conclusions can be derived about the molecular basis of fertility gene function.

The mild defects of *Y* chromosomal mutations *in cis* stand in sharp contrast to those of certain male-sterile mutations on other chromosomes, that interfere *in trans* with the formation of the lampbrush loop pairs by a wild-type *Y* chromosome. These mutations have been isolated in both *D. melanogaster* and *D. hydei* (Kiefer 1973; Lifschytz 1974, 1975; Hackstein et al. 1987,1990; Hackstein 1991), leading to a developmental arrest before or immediately after meiosis. For example, in *D. melanogaster* males carrying the *X*-linked mutation *downy*, the unfolding of all loop pairs seems to be blocked, and spermatogenesis does not proceed beyond early postmeiotic elongation stages (Kiefer 1973). A similar phenotype was described for *D. hydei* males carrying the *X*-linked mutation *ms(1)XL24* (Lifschytz 1975). The temperature-sensitive male-sterile mutation *ms(3)5* of *D. hydei* also prevents the unfolding of all lampbrush loop pairs at a restrictive temperature of 26°C, leading to a developmental arrest of spermatogenesis before meiosis (Hackstein et al. 1987). In addition, mutations are known in both species that specifically affect only one loop pair *in trans*, also leading to an early arrest of spermatid differentiation (Hackstein 1991).

4.6 Do the fertility genes encode proteins?

Ayles et al. (1973) isolated eigth temperature-sensitive (ts) mutations in the *Y* chromosomal fertility genes of *D. melanogaster*. The relation of these mutations to

the *kl* and *ks* complementation groups is unclear, but at least one of them (*ms(Y)B119*) is an allele of the loop-forming gene *kl-5* (K. Livak, cited by Goldstein et al. 1982). Other ts-mutations were tentatively assigned to genes *kl-1* and *kl-2*, which do not form a loop, but none was assigned to the loop-forming gene *kl-3*. In *D. hydei*, *Y*-linked ts-mutations have been isolated by Leoncini (1977). Two of them were mapped at the loop-forming fertility gene *A*, another was assigned to gene *B*, which does not form a loop, and at least one was assigned to the loop-forming gene *Q* (Hackstein et al. 1982). The authors also discuss the possibility that ts-alleles also exist for the loop-forming gene *N*. The temperature sensitive periods of all these mutations are during the primary spermatocyte stage (Ayles et al. 1973; Leoncini 1977).

Conventionally, temperature-sensitive mutations are interpreted as the result of a missense mutation in a protein coding gene, causing the formation of a thermolabile protein, both in prokaryotes (Jokusch 1966; Wittman and Wittman-Liebold 1966) and in eukaryotes (Suzuki 1970). Therefore, it seems likely that the *Y* chromosomal fertility genes identified by ts-alleles are protein coding genes. Consistent with this interpretation, the lampbrush loops formed by ts-alleles in *D. hydei* have a normal loop morphology at both the permissive and the restrictive temperature (Leoncini 1977; Hackstein et al. 1982, also see Chapter 8 of this thesis), as expected from a point mutation in a protein coding gene. In addition, also other data indicate that certain male-sterile alleles are point mutations, as non-conditional, EMS-induced alleles of gene *A* of *D. hydei* can be reverted to wild-type by a second EMS treatment. EMS induces point mutations but also complex chromosome rearrangements. It is highly unlikely that deletions, translocations, or inversions can be reverted by chemical mutagenesis (Hackstein et al. 1982).

Which roles are performed by the proteins that are encoded by the loop-forming fertility genes? There are two possibilities. The proteins either have a regulatory function, *i.e.* they function in the control of gene expression, or they have a structural function as a sperm protein that is required only during the final stages of sperm morphogenesis, *i.e.* for individualization or for sperm motility.

Indirect evidence for a regulatory function comes from comparisons of the testis protein contents of *XY* and *X0* males of *D. hydei*. When separated by one-dimensional PAGE, sperm proteins of M_r= 155 000 and 55 000 are reduced in amount in *X0* testis, while a protein of M_r= 35 000 is completely absent (Hulsebos et al. 1983). The M_r= 55 000 proteins includes the tubulins, which are encoded by a gene family with all of its members on the autosomes, both in *D. hydei* (R. Brand and W. Hennig, unpublished observations) and in *D. melanogaster* (Kemphues et al. 1980; Mischke and Pardue 1982; Biajolan et al. 1984). The nature of the other two protein fractions is unknown, but the M_r= 35 000 protein is absent in *D. melanogaster*. Evidently, the M_r = 155 000 and 55 000 proteins are not encoded by the *Y* chromosome, and possibly this is also the case for the M_r= 35 000 protein . Thus, the *Y* chromosome exerts a regulatory influence on the accumulation of proteins encoded by genes on other chromosomes. The region responsible for this effect was mapped to the region containing fertility genes *O*, *P*, and *Q*. Gene *O* forms the loop pair *Clubs*, gene *Q* forms the *Nooses* loop pair.

The molecular basis for these *trans* effects of the *Y* chromosome on protein synthesis is completely unknown. However, since the tubulin genes of *D. hydei* have been cloned, it was possible to show that the level of tubulin mRNAs in the testis of *X0* males is not different from that in *XY* males (unpublished observations of R.C. Brand and W. Hennig, cited by Hennig 1987b). Thus, at least for the tubulins, the effect on gene expression seems to be exerted at the posttranscriptional level. Since there is a ts-allele of gene *Q* (*ms(Y)Q4^{ts}*, see Leoncini 1977; Hackstein et al. 1982; Hackstein and Hennig 1982), it is tempting to speculate that this gene encodes a protein that regulates the translation of the mRNAs for the three sperm protein fractions. Unfortunately, it has not been investigated whether the accumulation of the three protein fractions, and the amount of tubulin mRNAs, are dependent on the breeding temperature of males carrying *ms(Y)Q4^{ts}*.

Evidence for a structural role of putative proteins encoded by loop-forming fertility genes comes from the analysis of mutations of genes *kl-5* and *kl-3* of *D. melanogaster*, and of genes *A* and *N* of *D. hydei*. As discussed above, mutations in each of these genes all lead to the absence of the outer dynein arms in the sperm axoneme. Dyneins are large, multimeric protein complexes that function as ATP-fueled microtubule motors (reviewed by Porter and Johnson 1989). There are ts-alleles for gene *kl-5*, gene *A* and possibly also for gene *N*, suggesting that the protein product of these genes is either dynein, or a regulatory protein that is specifically involved in the synthesis of dynein or its incorporation into the axonemal microtubules.

For genes *kl-5* and *kl-3*, Goldstein et al. (1982) found a correspondence between gene dose and the amount of a high molecular weight sperm protein with the same mobility on PAGE gels as one of the dynein proteins from *Chlamydomonas* axonemes. In the ts-allele *ms(Y)B119* of *kl-5*, isolated by Ayles et al. (1973), the dynein-like protein is formed at both the restrictive and the permissive temperature, but only at the permissive temperature the outer dynein arms are present in the axoneme. Thus, the conditional mutation most likely causes a missense mutation in a dynein gene located at *kl-5*. At the restrictive temperature, the mutation does not interfere with dynein synthesis or stability. Only its incorporation into the microtubules of the sperm axoneme is affected.

Recently, Gepner and Hays (1993) provided evidence for the location of DNA sequences encoding a member of the dynein β-heavy chain gene family in the region of the *Y* chromosome containing *kl-5*. These authors cloned a fragment of 392 bp of genomic DNA by the polymerase chain reaction, using primers derived from conserved amino acid domains. Since dynein β-heavy chains are very large proteins (M_r > 300 000; see Porter and Johnson 1989), that are encoded by mRNAs of more than 14 kb (Gibbons et al. 1991; Ogawa 1991), only 3% of the coding sequences of dynein have been sequenced so far. It is unlikely that these sequences belong to a defective pseudogene, since the dynein gene is transcribed when the loop-forming genes are active, *i.e.* not in spermatogonia, but only during the primary spermatocyte stage. The transcripts containing the dynein sequences are present in the cytoplasm of primary spermatocytes of wild-type males, but not in males lacking the region of the *Y* chromosome containing the *kl-5* gene. However, it has not been shown that the sequences encoding dynein are located within the lampbrush

loop pair *A* that is formed by *kl-5* (see Chapter 9, and Hennig 1993 for discussion).

A protein coding function of the lampbrush loop-forming fertility genes would be consistent with the genetic experiments described in sections 2 and 3, showing that only one mutable function can be assigned to each fertility gene. A protein coding function would also explain why certain alleles of several of the loop-forming genes nevertheless form a loop pair of normal morphology.

However, even if the loop-forming genes are protein coding genes, the question remains unresolved why a such a conventional function requires an unconventionally large gene size, and an unconventional mode of gene expression, *i.e.* the formation of a giant lampbrush loop. The largest protein coding gene known in *Drosophila* is the maternal-effect gene *pumilio* on chromosome *3*, that is required for the establishment of the anterio-posterior polarity of the embryo. This gene has a size of 160 kb (Macdonald 1992), but even the smallest lampbrush loop pair of *D. hydei*, the *Nooses*, with a size of 260 kb (Grond et al. 1983), is larger than this largest protein coding gene of *Drosophila*, again emphasizing the mysterious nature of the loop-forming fertility genes.

5 THE MOLECULAR COMPOSITION OF THE LAMPBRUSH LOOPS

After having reviewed the number of the *Y* chromosomal fertility genes, their properties in genetic experiments, the correlation between fertility genes and lampbrush loop pairs, and the contributions of these genes to the differentiation of the male germ cell, it is time to address the following questions. What is the structure of the lampbrush loops? What contributes to the cytological appearance of the different loop pairs, what are the molecules that form the loop pairs, and how do these molecules interact?

5.1 The lampbrush loops are sites of RNA synthesis

Following the pioneering autoradiographic studies of J. Gall, H. Callan and associates on the lampbrush loops formed in amphibian oocytes (reviewed by Callan 1986), Hennig (1967) studied the *in vivo* incorporation of [^{3}H]-uridine by the lampbrush loop pairs of *D. hydei*. Previously, Meyer (1963) had presented histochemical evidence for the presence of RNA-protein complexes in these loops. In a series of carefully controlled pulse-chase labelling experiments, Hennig (1967) could demonstrate that the loop pairs *Threads*, *Clubs*, and also the *Cones* (the small round projections of the *Pseudonucleolus*, see Fig. 1)) are sites of RNA synthesis. Also the autosomes, the *X* chromosome and the nucleolus synthesize RNA during the primary spermatocyte stage (Hennig 1967; Meyer and Hennig 1974; Glätzer 1975, 1979). The autosomes and the loops each account for about 50% of the non-nucleolar RNA synthesis (Hennig 1967).

Using cytological criteria, such as loop morphology and the shape and the

position of the nucleolus, a subdivision of the primary spermatocyte stage, which in *D. hydei* lasts for many days, was possible (Hennig 1967). The rate of RNA synthesis in the loops was found to differ between the successive stages. The loops appeared to be dynamic structures, in which an equilibrium existed between the synthesis and the degradation of RNA.

Stage 0 is the first stage (20 hours). This stage represents the G1 and the S phase, during which the DNA is replicated. Stage 0 is morphologically very similar to the spermatogonial stage. There are no visible lampbrush loops.

Stage I (24 hours) is characterized by the gradual unfolding of the lampbrush loops from a position close to the nucleolus. The loops actively synthesize RNA.

Stage II (90 hours) is the most active stage in RNA synthesis. The loops are fully expanded. The loops now are a steady state system, in which RNA is newly synthetized, stored for approximately 20-30 hours, and subsequently degraded.

In Stage III (27 hours) the rate of RNA synthesis in the loops becomes less. The loops are nevertheless morphologically similar to Stage II, but now they seem to be mainly engaged in RNA storage and degradation.

Stage IV (4 hours) is a short stage during which the cell prepares for the meiotic divisions. Transcription in the loops is only about 20% of that at Stage II. The nuclear membrane disappears, and the loops are degraded. When the first meiotic division starts, some residual material from the loop pairs *Clubs* and *Pseudonucleolus* forms the only discernible remnants of the loops (Hennig 1967; Hess and Meyer 1968).

Most likely, the RNA is synthetized in the loops by RNA polymerase II, since specific inhibitors of its activity, such as actinomycin, lead to a rapid desintegration of all the lampbrush loop pairs *in vivo* (Meyer and Hess 1965). Thus, the integrity of the loops seems to depend on their transcriptional activity. When transcription stops at the end of the primary spermatocyte stage, or if it is experimentally repressed by actinomycin, the loop structure disintegrates.

5.2 The lampbrush loops are giant transcription units

The similarity between the intranuclear structures formed by the *Y* chromosome of *Drosophila* and the amphibian lampbrush loops was already alluded to by Meyer et al. (1961) in their first description of the loops of *D. melanogaster*. As reviewed by Callan (1986), it became clear in the early seventies that many of the amphibian lampbrush loops contained multiple transcription units, having either the same or opposite orientations along the DNA axis. How many transcription units are present within the *Drosophila* lampbrush loops?

Autoradiographic studies by Hennig (1967) revealed a sequential labelling of the loop pair *Threads* of *D. hydei*, with the thin end of the loop starting to incorporate the label. However, application of the chromatin spreading techniques developed by Miller and Beatty (1969) enabled a direct visualization of lampbrush loop transcription in wild-type males of *D. hydei* (Hennig et al. 1974b; Meyer and Hennig 1974; Glätzer 1979; Glätzer and Meyer 1981) and of *D. melanogaster* (Glätzer 1980). An almost complete spreading of the compact part of the *Threads* seems to have been

accomplished by Glätzer and Meyer (1981). In addition, using *T(X;Y)* males of certain constitutions, transcription in individual loop pairs could be studied in *D. hydei* (see Grond et al. 1983 for the *Nooses*; de Loos et al. 1984 for the *Threads* and the *Pseudonucleolus*; there are unpublished observations of R. Suijkerbuijk and W. Hennig for the *Clubs*). Unfortunately, such studies, using defined genotypes that allow unambiguous loop identification, have not been performed for *D. melanogaster.*

In these so-called "Miller-spreads", the lampbrush loops could be visualized as giant transcription units. As determined from the length of the loop DNA, the size of the *Nooses* loop, which was visualized in its entirety, was estimated at 260 kb (Grond et al. 1983). The *Threads* and the *Pseudonucleolus* were visualized only partially, but by "adding up all linear sections" of the nascent loop transcripts, the size of the *Threads* transcription unit was estimated between 500 and 1000 kb, and that of the *Pseudonucleolus* at 1500 kb (de Loos et al. 1984). Although these latter two estimates may not be as accurate as that for the *Nooses*, these size estimates are consistent with earlier light microscopic measurements of the length of the loops, either directly in squashed testis (Hess and Meyer 1968; Hennig et al. 1974b), or by the tracing of the DNA axis of the loops after staining with the DNA-specific fluorescent dye DAPI (Kremer et al. 1986). They are also consistent with the high mutation frequency of the loop-forming genes (section 2.3) and with the localization of noncomplementing, sterilizing breakpoints (section 3.2), which also are very suggestive of a large physical size.

The morphology of the transcripts of the different loop pairs and their spacing along the DNA axis were found to be specific for that particular loop pair. In transcripts of the *Threads* a thin, bush-like part close to the DNA axis and a more distal and thicker fibrillar part could be distinguised. The non-uniform thickness of the *Threads* transcripts were interpreted to indicate differences in RNA-protein association (also see section 5.3). Transcripts of the *Clubs* were found to be tangled in large bush-like conformations, lacking a fibrillar part (de Loos et al. 1984).

Especially the smallest loop pair of *D. hydei*, the *Nooses*, could be analyzed in considerable detail. It is the only loop pair of which an entire transcription unit could be visualized (Grond et al. 1983). It is likely that this particular loop is a fully developed loop from a Stage II or III primary spermatocyte nucleus, and not an incompletely expanded loop from an earlier stage (see also Chapter 2). A single *Nooses* lampbrush loop appeares as a single, 260 kb-long transcription unit (Fig. 3), with a gradient of nascent, growing loop transcripts along the DNA axis of the loop. Thus, the *Nooses* loop has a unique site for the initation of loop transcription. This was found for the *Threads* as well, but the *Pseudonucleolus* seemed to have both a major transcriptional initiation site, and additional initiation sites within the transcription unit that are less frequently used (see de Loos et al. 1984 for details). The *Nooses* transcripts have a complicated secondary structure (Fig. 4). Close to the DNA axis they are covered with granules, but more distally they are highly branched and granules are absent. The granules can also be seen in ultrathin sections of fixed testis (Grond et al. 1984).

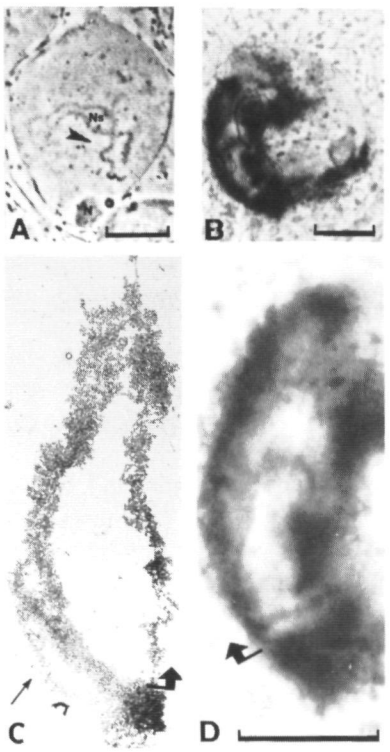


Fig. 3 A–D. Morphology of the lampbrush loop pair *Nooses*. **A** Primary spermatocyte nucleus of a *T(X;Y)58/0* male. Males of this genotype carry only the short arm of the *Y* chromosome, and therefore, primary spermatocyte nuclei display only the *Nooses* loop pair (*Ns*; indicated by the *arrowhead*). **B** Fully developed Stage II primary spermatocyte nucleus of a wild-type male after non-radioactive transcript *in situ* hybridization with a digoxigenin-labelled ay1 probe (see Chapters 2, 4 and 8). This probe specifically hybridizes to transcripts of the *Nooses*; the two loops can be distinguished. **C** In Miller spreads of transcribed chromatin of *T(X;Y)58/0* primary spermatocytes a complete *Nooses* lampbrush loop can be seen as a single transcription unit of 50 μm that contains an estimated 260 kb of DNA. The *solid, L-shaped arrow* indicates the approximate position of the initiation site for transcription. The *thin arrow* indicates a ribosomal transcription unit. From Grond et al. (1983). **D** One of the two *Nooses* loops from the Stage II primary spermatocyte shown in **B**, but now at the same magnification as the loop in **C**. Note the similarity in size and loop morphology, indicating that the spermatocyte nucleus used for the Miller spread was a Stage II or III nucleus, containing a completely unfolded *Nooses* loop pair. **A**, **B** and **C** phase contrast. All *bars* represent 10 μm

Thus, combining the data from sections 2, 3 and 5, the following relationship has been established for the lampbrush loop-forming fertility gene *Q* of *D. hydei*: **The lampbrush loop is a single transcription unit, being the cytological manifestation of a single genetic function that can be mutated to male sterility** (see also section 7.1). Most likely, this relationship also applies to those loop-forming genes for which the visualization of a complete transcription unit has not been possible because of their much larger sizes.

5.3 The lampbrush loops accumulate proteins not encoded by the *Y* chromosome

From the above, a lampbrush loop appears as an unfolded DNA axis that is actively transcribed. However, in the light microscope, neither the DNA axis, nor the RNA transcripts can be seen. What is seen, is protein, similar to the lampbrush loops of amphibian oocytes. Treatment of isolated *D. hydei* lampbrush loops with proteases (Hennig 1967), and histochemical studies of ultrathin sections or of squash preparations from whole testis, demonstrated that large amounts of proteins are present in the lampbrush loops (Hess and Meyer 1968; Yamasaki 1977, 1981). There were staining differences between the different loop pairs, and also in the electron

microscope loop-specific, granular, tubular, fibrillar or reticular structures could be seen, that were interpreted as loop-specific ribonucleoprotein complexes (Meyer et al. 1963; Grond et al. 1984). In the loop pairs *Threads*, *Pseudonucleolus* and *Clubs*, which do not appear homogeneous even at the level of the ligh microscope, the different regions of the loop were found to contain ribonucleoprotein complexes with different cytochemical staining properties. Since nucleic acids were detectable only in a few components of each loop, Grond et al. (1984) concluded that the loops mainly consist of protein.

Initially, it was proposed that the proteins were formed in the loops, *i.e.* that they were the gene products of the fertility genes themselves (Meyer and Hess 1965). This view, however, was proven to be incorrect. Because fertile hybrids between *D. hydei* and *D. neohydei* can be obtained (Hess and Meyer 1963a) it was possible to construct males carrying a *D. neohydei Y* chromosome, but with the *X* chromosome and autosomes of *D. hydei* (I. Hennig 1978; U. Schäfer 1978). The lampbrush loop pairs of such males are different from those of either parental species, indicating that at least some loop constituents are of autosomal origin. Indeed, using an immunological approach, Hulsebos et al. (1983;1984) and other investigators as well, showed that several of the lampbrush loop proteins of *D. hydei* are encoded by genes not located on the *Y* chromosome. As already mentioned in section 3.3, antisera raised against proteins from a variety of sources

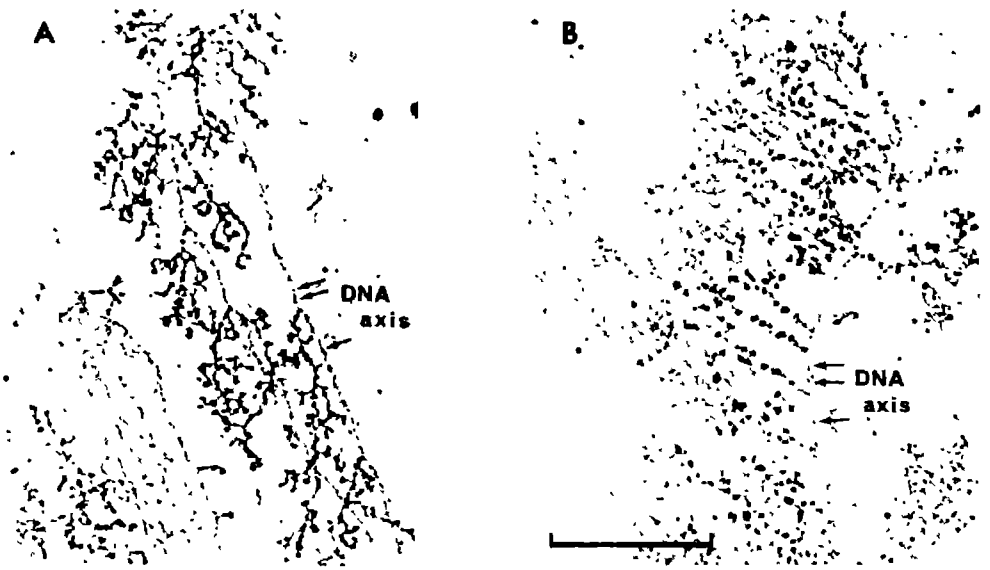


Fig. 4 A,B. Details of the transcribed DNA in the *Nooses* lampbrush loop of a *T(X,Y)58/0* male The DNA is wrapped in nucleosomes (indicated by the *arrows*). The *Nooses* transcripts have a complex, branched structure at their distal ends (**A**), whereas more proximally, they are covered with granules (**B**) From Grond et al. (1983). *Bar* represents 1 μm

were found to decorate specific loop pairs (Table 3). These antisera were raised against (i) nuclear proteins of a *D. melanogaster* embryo-derived cell line (Risau et al. 1983), (ii) cytoplasmic RNP protein of *D. melanogaster* (Schuldt and Kloetzel 1985) or *Xenopus laevis* (Dearsly et al. 1985), (iii) *D. melanogaster* histone H1 (Frasch et al. 1985), (iv) *Drosophila* sperm protein fractions isolated from protein gels (Hulsebos et al. 1983, 1984; Pisano et al. 1993), (v) a fusion-protein of *D. melanogaster* laminin B2 (Wang et al. 1992; Wang 1993), and finally, (vi) against specific components of the synaptonemal complex of rat spermatocytes (unpublished data of W. Hennig and C. Heyting; see Heyting.et al. 1989; Offenberg et al. 1991; Hennig 1993). Some of these antigens were also present during postmeiotic stages, when the loops have disappeared. For example, the *S5* antigen specifically decorates the protein body of the spermatid nucleus (Glätzer 1984), and so does an antiserum raised against histone H1 (Kremer et al. 1986). Tektin is found in the sperm tails (Pisano et al. 1993).

From the various immunological localization studies, summarized in Table 3, it can be concluded that in both *D. hydei* and *D. melanogaster*, different loop pairs contain different proteins, and that, again in both species, several proteins are present in more than one loop pair. Thus, differences in protein content seem to be the main reason for the morphological diversity of the loops. However, as discussed in the next section, this is not the only reason.

5.4 Do the lampbrush loop-forming genes function by protein binding?

From the finding that proteins are the major components of the lampbrush loop pairs, and from the identification of some of these proteins as sperm components, components of chromatin, components of heterogeneneous nuclear RNP, or components of the synaptonemal complex, it has been postulated that the biological function of the loop-forming fertility genes is to accumulate and store specific proteins (Hennig 1985; Hennig et al. 1989; Hennig 1990; Hennig and Kremer 1990; Gatti and Pimpinelli 1992). This hypothesis was earlier put forward by Callan (1982) for some of the lampbrush loops in amphibian oocytes. The loop proteins presumably perform important functions during spermatogenesis, as indicated by the isolation of male-sterile mutations in autosomal genes that modify the cytological appearance of a certain loop pair *in trans*, both in *D. hydei* (Hackstein et al. 1990) and in *D. melanogaster* (Hackstein 1991). Such mutations may identify genes that encode loop proteins. It has been suggested that the loops function as "nuclear storage organelles for RNP that is necessary for sperm development" (Glätzer 1984), or as "complex organelles devoted to the compartmentalization and processing of some proteins involved in spermatogenesis" (Pisano et al. 1993). In addition, their function may be "related to the process of chromosomal protein substitution" (Hennig 1990), that is reflected in the condensation-decondensation cycles of the chromatin during spermatogenesis (Kremer et al. 1986), or they may represent "a functional substitute for the synaptonemal complex" (Hennig 1993), as there is no meiotic recombination in the male.

Table 3. Antisera that decorate specific lampbrush loop pairs of *Drosophila*

antiserum	mono poly[1]	raised against[2]	decorated loop pair[3] in *D. hydei*	*D. melanogaster*	references[4]
sph155	p	M_r= 155 000 fraction testis proteins (*D.h.*)	*Ps*	*B*	1,2,3
tektin	p	M_r= 53 000 fraction testis proteins (*D.m.*)	*Ps*	*B*	4
histone H1	p	histone H1 (*D.m.*)	*Ps* *Cl* grana	n.d.	5
K7	p	M_r= 35 000 fraction testis proteins (*D.h.*)	compact *Th* *Tr*	n.d.	6
laminin B2	p	lacZ-laminin B2 fusion protein (*D.m.*)	*Ps* *Ns*	*A*	7,8
pp60	p	M_r= 60 000 pre-mRNP *Xenopus laevis* oocytes	*Ps* channels *Cones*, *Cl*, *Tr* compact *Th*	n.d.	9,10
Dm28K2	p	M_r= 28 000 cytoplasmic RNP (*D.m*)	*Ps* matrix *Cl*, *Cones*	n.d	9,10,11
Bv96	m	nuclear protein (*D.m.*)	*Ns*	n.d.	6
S5	m	nuclear RNP (*D.m.*)	diffuse *Th*, *Tr*	*A*, *C*	3,11,12,13
X4	m	nuclear RNP (*D.m*)	diffuse *Th*, *Tr*	*A*, *C*	11,12, 13
Knuf	p	M_r= 30 000 - 33 000 rat SC fraction	*Ps*	unidentified loop pair	14
Joe	p	M_r= 125 000 rat SC protein	*Cl* grana	unidentified loop pair	14
MabIX9D5	m	M_r= 190 000 rat SC protein	diffuse *Th*, *Tr*	n.d.	14

Notes

[1] both mono- (m) and polyclonal (p) antisera were used in these studies

[2] abbreviations: *D h* : *D. hydei*, *D m* : *D melanogaster*, RNP ribonucleoprotein, SC synaptonemal complex

[3] names of lampbrush loops are abbreviated *Ps Pseudonucleolus*, *Th Threads*, *Cl Clubs*; *Tr Tubular ribbons*; *Ns Nooses* (also see Fig 1), n d not determined

[4] References (1) Hulsebos et al 1983, (2) Hulsebos et al 1984, (3) Bonaccorsi et al 1988 (4) Pisano et al 1993, (5) H Kremer, cited by Hennig et al 1985, (6) Hennig 1990 (7) Wang et al 1992, (8) Wang 1993, (9) Glätzer and Kloetzel 1985, (10) Glatzer and Kloetzel 1986, (11) Glatzer and Bünemann 1987, (12) Glatzer 1984, (13) Melzer and Glatzer 1985, (14) unpublished observations of W Hennig and C Heyting, also see Offenberg et al (1991) and Hennig (1993)

The proteins were supposed to bind to the RNA transcripts of the loops (Grond et al. 1983, 1984; Glätzer 1984; Hulsebos et al. 1984; Kremer et al. 1986). As discussed in section 5.2, the loop transcripts have a loop-specific morphology. This may explain why different loops bind different proteins, and also why loops of different morphology contain immunologically related, or perhaps even identical proteins (Table 3). For example, the *Pseudonucleolus* of *D. hydei* and loop *B* of *D. melanogaster* are stained by antisera raised against tektin and against the *sph155* sperm protein, but they have an entirely different morphology and do not share transcribed DNA sequences (see section 6). In fact, the only two species with some morphological similarities of the lampbrush loops are *D. hydei* and its sibling species *D. neohydei* (Hess and Meyer 1963a; I. Hennig 1978, 1982), and these two species share most, but not all, of the DNA sequences transcribed in the loops (Table 4).

Thus, loop morphology is the result of loop-specific interactions between the loop transcripts and the loop proteins, as already argued by Hennig (1967). Indeed, a specific association between the *S5* antigen and transcripts of the *Tubular ribbons* and the diffuse part of the *Threads* could be demonstrated (Glätzer and Bünemann 1987). In a "Northwestern" experiment, [^{32}P]-labelled transcripts of the YLII family of repetitive DNA sequences (see section 6) bind to a testis protein fraction of a size corresponding to that of the antigen. In primary spermatocyte nuclei the position of this protein exactly coincides with that of the YLII containing transcripts of the *Tubular ribbons* and the diffuse part of the *Threads*.

The main attractions of the hypothesis that the loop-forming fertility genes function by protein binding are the following. First, the hypothesis explains why all sperm components are formed in *X0* males, and in addition, why mutations of the loop-forming genes have only subtle, "organizational" effects on sperm morphogenesis (see section 4.5). Second, it explains why the loop-forming genes appear to consist only of repetitive DNA sequences that have no protein coding potential (see section 6). Third, and most importantly, it explains why gene function requires the formation of a lampbrush loop pair.

However, there are several observations that are not easily compatible with the hypothesis that protein coding is the only function that can be mutated to male sterility. First, the hypothesis would be proven if it could be shown that a male-sterile mutation in a loop-forming gene prevents normal protein binding to the corresponding lampbrush loop. Nevertheless, at the level of the light microscope, several male-sterile alleles of loop-forming fertility genes in *D. hydei* form cytologically normal loop pairs (Leoncini 1977; Hackstein et al. 1982, 1991). In lampbrush loop pairs formed by certain sterile alleles of fertility genes *kl-5* and *ks-1* of *D. melanogaster*, the staining pattern of the *S5* antigen is normal, and so are the staining patterns of both *sph155* and tektin in certain sterile alleles of gene *kl-3* (Bonaccorsi et al. 1988). Thus, it seems reasonable to assume that the major protein constituents are present in these loops, and also that they are present in approximately normal amounts. It can be concluded therefore, that the mutational event that destroys gene function does not in all cases detectably interfere with the binding of the abundant loop proteins. In addition, mutations of genes *kl-5* and *kl-3* (or both) always cause the absence of the outer dynein arms, irrespective of

loop formation. Thus, irrespective of whether protein binding is possible, the male-sterile phenotype is similar, even at the level of the electron microscope.

Second, deletions of lampbrush loop-forming genes neither affect the migration of loop-proteins into the primary spermatocyte nucleus, nor do they affect the postmeiotic distribution of loop proteins. In absence of both gene *A*, forming the *Threads*, and gene *N*, forming the *Tubular ribbons* in *D. hydei*, the *S5* antigen enters the nucleus and it is found at its normal postmeiotic position (Hackstein et al. 1991). In absence of gene *kl-3* of *D. melanogaster*, forming loop pair *B*, tektin does not enter the nucleus, but it is found at its normal position in the sperm tails (Pisano et al. 1993). Thus, the compartmentalization and storage of a protein on a lampbrush loop before meiosis is not in all cases required for its proper postmeiotic localization.

Third, if the binding of specific proteins to a loop is essential for the execution of sperm morphogenesis, it is expected that deletions of loop-forming genes of *D. hydei* and *D. melanogaster* that contain immunologically identical proteins, cause a similar male-sterile phenotype. However, this is not the case. The loop pair *Pseudonucleolus*, formed by fertility gene *B* of *D. hydei*, and the loop pair *B*, formed by gene *kl-3* of *D. melanogaster*, are stained by the same antisera (Table 3), but the phenotypic consequences of deletions of the corresponding fertility genes are not the same. Deletions of *kl-3* cause absence of the outer dynein arms in the axoneme (Hardy et al. 1981), but in males deficient for gene *B*, the outer dynein arms are present (N. Kociok, cited by Hackstein et al. 1991).

Fourth, it has not been shown for any lampbrush loop protein of *D. hydei* or *D. melanogaster* that immunologically related proteins are present in the lampbrush loops of most *Drosophila* species. This would be expected if protein binding is the conserved function of the loop-forming genes. In the most extensively studied case, Hulsebos et al. (1984) determined for 22 species whether they have a lampbrush loop pair that is decorated with the polyclonal *sph155* antiserum, raised against a *D. hydei* sperm protein. However, 10 of these species lack an immunologically related protein in their lampbrush loops, and in 6 of the 12 species displaying loop staining, the antigen is not detectable in postmeiotic stages. The occurrence of the *sph155* loop protein does not follow the phylogenetic relations between the 22 species studied, either established by immunological distances of the larval hemolymph protein LSP2 (Beverly and Wilson 1984), by cladistic analyses of two-dimentional gel electropheresis patterns of 135 abundant proteins (Spicer 1988) and of 217 morphological criteria (Grimaldi 1990), or by the divergence of protein coding DNA sequences as determined by hybridization of complementary DNA (Caccone et al. 1992).

Thus, it is clear that the transcripts of the lampbrush loop pairs of *Drosophila* have the property to accumulate proteins, but the relation between protein binding and fertility gene function is not clear. Access to the nature and function of the loop proteins may be provided by male-sterile mutations that cause modifications of loop morphology *in trans*, as such mutations may directly identify genes encoding loop proteins (Hackstein et al. 1990; Hackstein 1991).

6 THE MOLECULAR COMPOSITION OF LAMPBRUSH LOOP-FORMING GENES

The next question to be addressed concerns the nature of the DNA sequences that are transcribed in the lampbrush loop-forming male fertility genes. As will be discussed in this section, this question can be answered only indirectly, since so far, all conclusions on the molecular organization of the DNA sequences in the loops are based on pieces of cloned *Y* chromosomal DNA of a few kb, that hybridize to the giant loop transcripts Because of the repetitive nature of all these cloned DNA sequences, it is not absolutely certain that the cloned pieces are indeed located within the loop-forming transcription units Data from the analysis of cDNA clones are very sparse (see section 7) It will be shown in this section that the sequence analysis of DNA clones has not revealed the basis for the function of the loop forming genes

6.1 Isolation of DNA from the *Y* chromosome

6.1.1 *D. hydei*

The autoradiographic studies of Hennig (1967) had indicated that the *Y* chromosomal lampbrush loops of *D hydei* synthesize about 50% of the non-nucleolar RNA during the primary spermatocyte stage Subsequently, Hennig (1968) showed that labelled RNA extracted from testis, but not from the somatic tissues of *XY* males, was able to hybridize to immobilized genomic DNA from *XY* or *XYY* males, even in the presence of a 20-fold excess of unlabelled competitor RNA The experimental conditions were such that only RNA transcribed from repetitive DNA sequences could hybridize It was therefore concluded that transcribed DNA sequences from the *Y* chromosome are repetitive

Efforts to isolate repetitive *Y*-specific DNA sequences of *D hydei* by analytical density centrifugation were unsuccessful (Hennig et al 1970, Hennig 1972, Renkawitz 1978a,b) The highly reiterated DNA sequences isolated by these procedures hybridized to the *Y* chromosome as well as to other chromosomes, such as the *X* chromosome

With the advent of recombinant DNA technology, the conclusion that the *Y* chromosomal DNA is repetitive gained general support The first report describing clones containing *Y*-specific DNA sequences was published by Lifschytz (1979) He screened duplicate filters containing lysed bacterial colonies of a genomic EcoRI library in plasmid vectors with labelled, total genomic DNA from males and from females, allowing the discrimination of clones containing *Y*-specific DNA Vogt et al (1982) used a PstI plasmid library that was screened with a similar procedure, but now using more sensitive hybridization to dots of purified plasmid DNA instead of total DNA from lysed bacterial colonies Using either procedure, the fraction of *Y*-specific clones was much less than expected on basis of the size of the *Y* chromosome, which represents 10% of the diploid male genome (Zacharias et al 1982) Only 3% (Lifschytz 1979, Lifschytz et al 1983) or 2% (Vogt and Hennig 1983) of the clones exclusively consisted of *Y*-specific DNA sequences. The relevant information about the cloned loop constituents is summarized in Table 4.

The *Y* chromosomal origin of the cloned DNA fragments was verified by comparing the hybridization patterns to Southern blots of genomic DNA from males and females. Following this approach, most clones were found to hybridize to DNA from both sexes (Vogt and Hennig 1983). Such clones appeared to consist of an *Y*-specific sequence, causing the hybridization to male-specific restriction fragments, and of a sequence causing the hybridization to fragments shared between both sexes. Shared sequences were named *Y*-associated (Vogt and Hennig 1986a). By *in situ* hybridization to neuroblast (pro)metaphase chromosomes, or by hybridization to Southern blots of genomic DNA prepared from *T(X;Y)* males that carry only a part of the *Y* chromosome, the cloned DNA sequences

Table 4. Families of repetitive DNA sequences transcribed in *D. hydei* lampbrush loops

family	loop pair[1]	sequence complexity[2]	sequence conservation[3]	also transcribed in[4] *neo*	*eo*	*bif*	*vir*	*mel*	references[5]
rally	*Th* , *Ps*	200 bp	95%	-	-	-	-	-	1,2
YLI	*Th*^{c}, *Tr*	700 bp	85%	+	+	-	-	-	3,4
YLII	*Th*^{d}, *Tr*	77 bp	85%	+	+	-	-	-	3,4
micropia	*Th*, *Ps*	5.5 kb	80%	+	+	-	-	-	5,6
YLIII	*Ps*, *Cones*[6]	GTCT	n.r.	+	+	+	+	-	3,4
$(CA)_n$	*Cones*	CA	n.r.	+	+	n.d.	n.d.	+	7,8
$(GT)_n$	*Ps*	GT	n.r.	n.d.	n.d.	n.d.	n.d.	n.d.	7,8
YDh22	*Tr*	73-55-57bp[7]	70-80%	+	+	+	-	-	9,10
YDh18	*Cl*	GATTGAT	n.r.	+	+	-	-	-	9,10
ay1[8]	*Ns*	400 bp	85%	+	+	-	-	-	3,4,9 to14
YsI[9]	*Ns*	600 bp	85%	+	+	-	-	-	3,4,9,10,11

Notes:

[1] Abbreviations are *Th*: compact part of *Threads*; *Th* : diffuse part of *Threads*; *Ps*: *Pseudonucleolus*; *Tr*: *Tubular ribbons*; *Cl*: *Clubs*; *Ns*: *Nooses*

[2] bp: basepairs; kb: kilobasepairs

[3] Average percentage sequence identity between two repeats of *D. hydei*; n.r.: not relevant for the simple repeat sequences

[4] Abbreviations of *Drosophila* species are: *neo*: *D. neohydei*; *eo*: *D. eohydei*; *bif*: *D. bifurca*; *vir*: *D. virilis*; *mel*: *D. melanogaster*. With the exception of the latter two species, all these species belong to the *repleta* group. n.d.: not determined

[5] References are: (1) Huijser and Hennig 1987; (2) Trapitz et al. 1992; (3) Wlaschek et al. 1988; (4) Trapitz et al. 1988; (5) Huijser et al. 1988; (6) Lankenau 1993; (7) Huijser et al. 1987; (8) Huijser et al. (1990); (9) Lifschytz et al. 1983; (10) Hareven et al. 1986; (11) Lifschytz and Hareven 1985; (12) Vogt et al. 1982; (13) Vogt and Hennig 1986a; (14) Vogt et al. 1986

[6] One strand of YLIII is transcribed in the *Pseudonucleolus*, the other in the *Cones*

[7] These different sequence blocks can be arranged in many different ways

[8] This family was named YDh23 in reference (8); Y23Ns in (9) and (10), and YsIa in (3) and (4)

[9] This family was named YDh20 in reference (8) and Y20Ns in (9) and (10). It is an evolutionary derivative of ay1, and claimed to be transcribed in the *Nooses* in references (2), (9) and (11), and claimed not to be transcribed in reference (4). This issue is settled in Chapter 4 of this thesis

could be mapped to a defined region of that chromosome. By *in situ* hybridization to polytene chromosomes from salivary gland cells, clones hybridizing to genomic DNA from females on Southern blots were found also to hybridize to positions in the euchromatin.

An important next step was to show that the cloned DNA fragments hybridized to transcripts of the loop-forming fertility genes. The clones isolated by Vogt et al. (1982) and Vogt and Hennig (1983) hybridized to the *Nooses*, including subcloned *Y*-associated DNA fragments (Vogt and Hennig 1986b). The *Y*-specific family of repetitive DNA sequences that hybridize to *Nooses* transcripts was named ay1 (Vogt and Hennig 1986a). It is identical to the YDh23 family of Lifschytz et al. (1983). In addition, these authors identified *Y*-specific clones that hybridized to transcripts of the loop pairs *Clubs* and *Tubular ribbons*. On Northern blots, ay1 and the other *Y*-specific sequences hybridized exclusively to total RNA from testis, not to RNA from the somatic parts of the male flies, neither to RNA from females, again confirming their *Y* chromosomal origin (Vogt et al. 1982; Lifschytz et al. 1983).

Thus, already the first detailed investigations of the molecular composition of the *Y* chromosome showed that the *Y* chromosomal DNA is a mosaic of repetitive DNA sequences, some of which are *Y*-specific, and others which also occur on other chromosomes. The assigments of a clone to a particular loop pair by transcript *in situ* hybridization were consistent with the hybridization of that clone to the approximate position of the corresponding loop-forming gene on the metaphase *Y* chromosome (see sections 2 and 3). As expected from the large transcript sizes seen in the Miller spreading experiments (section 5.3), the DNA sequences from the loops did not hybridize to a specific RNA species on Northern blots, but to a smear of heterogeneous transcript sizes.

These conclusions have been confirmed and extended by the analysis of *Y* chromosomal DNA sequences that were isolated by other procedures. Hennig et al. (1983) used a microcloning technique for the direct cloning of lampbrush loop DNA. They extracted DNA from manually isolated *Threads* loop pairs, digested the DNA with EcoRI and used it for cloning. The DNA fragments obtained hybridized to transcripts of the loop pair of origin, to the position of the *Threads* on the (pro)metaphase *Y* chromosome, and in addition, to positions on other chromosomes. This experiment yielded the "DhMiF" series of clones, including clones DhMiF2 and DhMiF8, that were identified by Huijser et al. (1988) as defective retrotransposons of the new *micropia* family. Such elements are also present in the loop pair *Pseudonucleolus*. *Micropia* probes hybridize to a heterogeneous smear of transcripts on Northern blots of testis RNA from *XY*, but not from *X0* males, and only the sense strand is present in loop transcripts (also see S. Lankenau et al. 1994).

A similar microcloning experiment was conducted for the *Pseudonucleolus* loop pair, again using EcoRI for DNA digestion (Huijser 1987). This experiment yielded the "DhMiP" series of clones. The *Y*-specific *rally* family was found to be a constituent of the *Pseudonucleolus*, and also of the proximal diffuse part of the *Threads* loop pair. This family, with a basic repeat length of 200 bp, corresponded to a 200 bp segment from the coding region from the gene for 28S ribosomal

RNA. In the loop DNA the 200 bp repeats appear to be organized in long stretches of tandem repeats (Huijser and Hennig 1987).

In addition, several of the DhMiP clones were found to contain long runs of the simple sequence 5' $(CA)_n$ 3', where n has values up to 40. Using [^{3}H]-labelled $(CA)_n$ or $(GU)_n$ RNA probes, Huijser et al. (1990) could show that $(CA)_n$ is intensively transcribed in the *Cones*, the small projections of the *Pseudonucleolus* (see Fig. 1), and much weaker in the *Pseudonucleolus* as well. $(GT)_n$ is also transcribed in the *Pseudonucleolus* but not in the *Cones*. This sequence, however, is not *Y*-specific, since it occurs throughout the genome (Huijser et al. 1987).

It is obvious that the success of the cloning procedures described above is highly dependent on the cleavage frequency of the particular restriction enzymes in the target DNA. Since in these experiments for practical reasons six-cutters were used (PstI and EcoRI), the recognition site will occur, on the average, once in approximately every 4 kb of DNA. Since many of the repetitive DNA sequences on the *Y* chromosome have a much smaller repeat length (Table 4), they may not contain the recognition sequence.

In an effort to circumvent this limitation, Awgulewitsch and Bünemann (1986) used the four-cutter enzyme Sau3A for the construction of genomic libraries. The DNA used for cloning was enriched for *Y*-chromosomal DNA sequences by the repeated cycling of DNA from males through chromatographic columns loaded with DNA from females. Obviously, this procedure strongly selects against the recovery of *Y*-associated DNA sequences. However, the employment of Sau3A enabled the identification of three new families of repetitive DNA sequences, named YLI, YLII, and YLIII (Wlaschek et al. 1988; also see Table 4). YLII and YLIII are *Y*-specific, but YLI also occurs in the proximal *X* heterochromatin. These families escaped detection in the microcloning experiments since they are poor in EcoRI sites (Trapitz et al. 1988,1992). Also members of the *Nooses*-specific ayl family, named YsIa by Wlaschek et al. (1988), were recovered, as well as members of the YsI family, that was shown to be an evolutionary derivative of the ayl family. YsI is identical to the YDh20 family of Lifschytz et al. (1983), and is also believed to be transcribed in this loop pair (Lifschytz and Hareven 1985).

By transcript *in situ* hybridization, YLI was found to be transcribed in the compact part of the *Threads*, and also in the *Tubular ribbons*, YLII was transcribed in the diffuse part of the *Threads*, and in the *Tubular ribbons* as well. Since in these experiments strand-specific probes were used, it was also shown that all members of a given family have the same orientation within the lampbrush-loop forming transcription unit, as it was earlier shown for the *Nooses*-specific ayl repeats by Lifschytz and Hareven (1985). For YLIII it was shown that one strand is transcribed in the *Pseudonucleolus*, and the other in the *Cones* (Trapitz et al. 1988,1992; Trapitz 1992).

In summary, DNA sequences hybridizing to transcripts of each of the five lampbrush loop pairs of *D. hydei* have been cloned. From the analysis of these clones, it can be concluded that the DNA that is transcribed in each loop pair is for a large part composed of *Y*-specific, repetitive DNA sequences. In the loop pairs *Threads*, *Pseudonucleolus* and *Nooses*, the *Y*-specific repetitive DNA sequences are interspersed by sequences that also occur on other chromosomes, so-called

Y-associated sequences. A given repeat family may be transcribed in one, or in two loop pairs, but within a loop-forming transcription unit all members of a family have the same orientation. YLIII and $(CA)_n$ may be an exception to this rule: opposite DNA strands are transcribed in *Cones* and *Pseudonucleolus*. Whereas it is unknown if both structures are formed by the same transcription unit, the *Cones* could not be assigned to a separate fertility gene (Hackstein 1987; Hackstein et al. 1991).

6.1.2 *D. melanogaster*

The molecular composition of the lampbrush loop-forming genes of *D. melanogaster* started to become a matter of interest almost a decade after the first DNA sequences from the loops of *D. hydei* had been identified. The initial investigations into the molecular organization of the *D. melanogaster Y* chromosome were focussed on the localization of the genes for ribosomal RNA (reviewed by Ritossa 1976), and of the various types of satellite DNA sequences isolated from CsCl density gradients by analytical centrifugation (reviewed by Peacock et al. 1973), but not on the molecular composition of the *Y* chromosomal fertility genes. However, these two lines of investigation recently merged (Bonaccorsi et al. 1990; Bonaccorsi and Lohe 1991; Gatti and Pimpinelli 1992).

A fairly detailed picture of the localization of various pentameric and heptameric satellite DNA sequences on the *Y* chromosome was presented by Peacock et al. (1977), who estimated that about 73% of this chromosome is represented by ribosomal DNA and by five different satellite DNA sequences. None of these sequences, however, is *Y*-specific, as ribosomal DNA also occurs in the *X* heterochromatin and the satellite DNA sequences are also found in the *X* heterochromatin and the centromere-associated heterochromatin of the autosomes.

The first DNA sequence shown to be specifically transcribed in a lampbrush loop pair of *D. melanogaster* was $(CA)_n$. This sequence was found to be transcribed in a lampbrush loop pair in all *Drosophila* species tested (Huijser et al. 1987, 1990). It is not known in which of the three loop pairs of *D. melanogaster* the $(CA)_n$ sequences are transcribed.

Bonaccorsi et al. (1990) used the different satellite DNA sequences cloned by Lohe and Brutlag (1986) for *in situ* hybridization on lampbrush loop transcripts. Hybridization conditions were carefully controlled in order to prevent cross-hybridization between pentameric or heptameric repeat sequences differing at only one nucleotide position. Together with other results, mentioned by Gatti and Pimpinelli (1992) it appeared that repeats of the sequence 5' AAGAC 3' and 5' AAGAG 3' were transcribed in loop pairs *A* and *C*, whereas repeats of the sequence 5' AATAT 3' were transcribed in loop pair *B*. It is not known which strand of these sequences is transcribed. On Northern blots of total testis RNA from *XY* males, but not from *X0* males, a nick translation-labeled 5' AAGAC 3' probe hybridized to a heterogeneous smear of transcripts, as was earlier found for the transcribed repeats of *D. hydei*. It was concluded that major parts of the DNA that is transcribed in the loops contain long arrays of these simple sequences.

It is not known whether additional satellite sequences are transcribed in the lampbrush loop pairs of *D. melanogaster*. A precise assignment of eight different cloned satellite DNA sequences to certain regions of the *Y* chromosome was accomplished by Bonaccorsi and Lohe (1991) by the use of *T(X;Y)*s and *T(Y;A)*s previously characterized by Bonaccorsi et al. (1988). This study confirmed the earlier results of Peacock et al. (1977), who used uncloned satellite DNAs as a probe. The most important result was that the cytogenetic location of each fertility gene (Fig. 1) coincided with the hybridization signals of certain combinations of satellite DNA sequences, including the pentameric repeats mentioned above, but also additional satellite repeats. Unfortunately, this studies was not extended by transcript *in situ* hybridization experiments, and satellite repeats hybridizing to regions containing fertility genes were also localized in regions of the *Y* chromosome not involved in fertility gene function. With respect to the sequence content of the loop-forming genes, the study of Bonaccorsi and Lohe (1991) is therefore inconclusive.

In summary, each of the *Y* chromosomal fertility genes of *D. melanogaster* contains a gene-specific combination of highly repetitive DNA sequences, similar to the situation in *D. hydei*. However, in contrast to *D. hydei*, all these sequences are *Y*-associated because they also occur in other heterochromatic regions of the genome. In addition, *Y*-specific lampbrush loop sequences have so far not been identified, and *Y*-associated DNA sequences from loop-forming genes that also occur in the euchromatin, such as transposable elements, have also not been reported in *D. melanogaster*.

6.2 Sequence analysis of cloned *Y* chromosomal DNA

Sequencing studies of transcribed *Y*-specific repetitive DNA of *D. hydei* did not reveal any protein coding potential (Vogt and Hennig 1986a,b; Lifschytz and Hareven 1985; Huijser and Hennig 1987; Lifschytz 1987; Wlaschek et al. 1988). Such studies revealed only the internal repetitive substructures of the repeats. For instance, the 700 bp YLI repeat is composed of an A subunit of 180 bp and three B subunits of 171 bp. In addition, it was found that the different families displayed varying degrees of sequence conservation. For example, repeats of the *rally* family shared 95% similarity, but repeats of the ay1 family only 85%. A comparison of 25 repeats of the YLII family, which have a sequence complexity of 77 bb, did not allow the construction of a consensus sequence, since only 28 nucleotide positions were conserved between all repeats (Wlaschek et al. 1988). Furthermore, it was apparent that members of the ay1, YsI, YLI, YLII and *rally* families contained deletions and duplications.

The *Y*-associated *micropia* retrotransposons, that are transcribed in the loop pairs *Threads* and *Pseudonucleolus* of *D. hydei* have lost the ability to encode the retroviral-like proteins (Huijser et al. 1988). Two members (DhMiF2 and DhMiF8) have been sequenced (see also D.-H. Lankenau et al. 1989, 1990). DhMiF8 has been subject to deletions and to insertions of other transposable elements (also see Chapter 7).

In summary, the DNA sequences identified as constituents of the lampbrush loop-forming fertility genes of *D. hydei* and *D. melanogaster* do not encode proteins. Obviously such a function cannot be performed by simple satellite DNA sequences or $(CA)_n$ repeats, but also the more complex repetitive sequences and the *Y*-associated retrotransposons have no large open reading frames. The different members of the more complex families display varying degrees of sequence divergence.

6.3 Implications for models for the functions of the loop-forming genes

It has been argued in section 4 that, given the apparent conservation of spermatogenesis, also the molecular mechanisms of its genetic regulation are likely to be conserved. On this basis, it has been postulated that the functions of the *Y* chromosomal lampbrush loop-forming fertility genes are conserved among the different *Drosophila* species, and this hypothesis is confirmed by the similarity of the male-sterile phenotypes caused by mutations of such genes in *D. hydei* and *D. melanogaster* (see section 4.5).

However, *D. melanogaster* and *D. hydei* do not share any of the sequences that are transcribed in lampbrush loop-forming genes, with $(CA)_n$ as the only exception (Huijser et al. 1987, 1990). In fact, the sequence 5' AAGAC 3' from *D. melanogaster* is even completely absent in the sibling species *D. simulans*, which does not have detectable amounts of such repeats in its genome (Lohe and Brutlag 1987), and the *rally* sequence of *D. hydei* is undetectable in the sibling species *D. neohydei* (Huijser and Hennig 1987). Similarly, with the possible exception of $(CA)_n$, the other transcribed *Y*-specific sequences of the *D. hydei* loops are absent in 69 of the 72 species assigned to the *repleta* group by Wasserman (1982), as they have been found only in *D. neohydei* and the somewhat more distantly related species *D. eohydei* (see Table 4). Also the *Y*-associated sequences of *D. hydei*, such as the *micropia* retrotransposons, are transcribed in the *Threads*-like loop pair of *D. neohydei*, but not in any of the loop pairs of *D. melanogaster* (D.-H. Lankenau 1993; S. Lankenau et al. 1994), although this species contains *micropia* elements in its genome (D.-H. Lankenau et al. 1989; D.-H. Lankenau 1990).

Thus, the majority (if not all) of the DNA sequences that are transcribed in lampbrush loop-forming fertility genes lack any conservation, even between closely related species. Therefore, these genes evolve at a much faster rate than the rest of the genome. From the assumption that the conserved function of the loop-forming fertility genes is based on conserved DNA sequences, it would follow that it is highly unlikely that the rapidly evolving, transcribed sequences, which are not even shared between sibling species, are important for this conserved gene function. But can they nevertheless function in protein binding? Usually, interactions between proteins and nucleic acids require short sequence motifs of about 10 nucleotides or even less. It is not impossible therefore, that even in those families with observed rates of 15–30% sequence divergence between the various members, such short sequence motifs are conserved, and that these short, but

functionally important motifs are present in the loop DNA of many *Drosophila* species. However, the available sequence data are too limited to allow the recognition of such sequence motifs. It has not been attempted to identify testis proteins that bind to transcripts of the sequence $(CA)_n$, that occur in the primary spermatocyte nuclei of all *Drosophila* species investigated (Huijser et al. 1987, 1990).

From these considerations it follows that, so far, the molecular data have been inconclusive with respect to the functions of the loop-forming fertility genes. Protein coding sequences or conserved sequence motifs for the binding of specific testis proteins have not been identified in the DNA that is transcribed in the loops. It should be kept in mind however, that all alleles of the loop-forming genes that do not unfold the corresponding lampbrush loop pair, are sterile. Thus, whatever the function of the transcribed repetitive DNA sequences may be, it can be concluded that their transcription in a megabase-sized lampbrush loop during meiotic prophase is required for male fertility. This conclusion was the basis for the work described in this thesis.

7 THE PURPOSE OF THIS THESIS

The studies of short pieces of DNA from the *Y* chromosome have indicated that the loop-forming fertility genes mainly consist of repetitive DNA sequences. However, information on the long-range organization of these sequences within the loop-forming transcription units is lacking. Such information cannot be derived from cloned pieces of *Y* chromosomal DNA with a size of only a few kb. In the best studied case, that of the *Nooses* loop pair of *D. hydei*, such clones can only represent less than 10% of the loop-forming transcription unit. In the cases of other loop pairs, as for example the *Clubs* and the *Tubular ribbons*, the cloned pieces of DNA represent less than 1% of the loop. With the exception of clones recovered from microdissected loop material, all clones were isolated by procedures designed to select for *Y* chromosomal DNA sequences, not for DNA sequences transcribed in the lampbrush loop-forming genes. Therefore, it cannot be excluded that the cloned sequences are not located within the loop-forming transcription units.

7.1 Strategies for cloning the DNA transcribed in the lampbrush loops

The most direct way to isolate transcribed DNA sequences of a certain gene is by the screening of cDNA libraries prepared from tissues in which the gene is known to be expressed. At first sight, the isolation of cDNA clones corresponding to DNA sequences from the loops should be feasible, since genomic clones, containing repetitive DNA sequences that hybridize to the transcripts of one or two loop pairs of *D. hydei*, are available (Table 4). When these clones are used to prepare probes

for hybridization to Northern blots of total testis RNA, the signals seem to be sufficiently strong to permit the recovery of cDNA clones. Following this approach, Papenbrock (1991) has isolated 10 different cDNA clones. Six clones belonged to the ay1 family, three to the YLII family and one to the YLIII family.

However, this approach has two disadvantages. First, transcripts of the fertility genes are not polyadenylated (Lifschytz et al. 1983; Wlaschek et al. 1988; see also Chapter 5), and therefore, cDNA clones corresponding to loop transcripts can be isolated only by the screening of cDNA libraries that have been prepared from total testis RNA. This necessitates the screening of enormous numbers of clones, because in total RNA, about 95% is ribosomal RNA. Papenbrock (1991) screened only a total of 250,000 λGEM11 bacteriophage plaques, and therefore the efficiency of the cloning procedure is very low (0.04%). A second disadvantage is that only short clones could be recovered. The longest cDNA had an insert of 1319 bp, and the average insert size of the 10 cDNA clones was approximately 540 bp (Papenbrock 1991).

Thus, whereas cDNA cloning identifies transcribed sequences from the loops, it does not give insight in the long-range organization of these sequences within the loops. For the work described in this thesis a different strategy was therefore adopted, aimed at the direct isolation of the genomic DNA that is transcribed in one of the loop pairs of *D. hydei* (Fig. 5). For this approach the *Nooses* lampbrush loop pair was selected, that is formed by fertility gene *Q* on the short arm of the *Y* chromosome. The reasons for this choice were the following.

(1) Gene *Q* has been intensively studied at the genetic level: The genetic studies have shown that there is one fertility gene, named gene *Q*, on the short arm, identifying a unique function that is indispensable for male fertility, as can be deduced from the following observations

Six male-sterile alleles of gene *Q* (*ms(Y)Q1* to *ms(Y)Q6*), one of them temperature-sensitive, have been induced by EMS on a wild-type *Y* chromosome (Leoncini 1977, Hackstein et al. 1982, Hackstein and Hennig 1982), and one sterile allele was induced by spontaneous transposition into the short arm of the *white-mottled-Confluens* giant transposable element (J. H. P. Hackstein, personal communication; also see Chapter 8). These mutations were mapped on the short arm by their failure to complement *T(X,Y)54*, *55*, *56*, and *57*, four different *X-Y* translocations that carry all the fertility genes of the long arm. In addition, they were all complemented by 18 different *T(X,Y)*s, carrying the short arm of the *Y* chromosome, that were induced by Hess (1965b) and Hackstein et al. (1982).

All combinations of the sterile alleles in *X/ms(Y)Qi ms(Y)Qj* constitutions failed to complement (Hackstein et al. 1982, J. H. P. Hackstein personal communication). Since *D. hydei* males carrying two copies of a wild-type *Y* chromosome are fertile (Hess and Meyer 1963a), and since it is unlikely that all these alleles are identical, they seem to identify one complementation group. However, it could not be excluded that the sterile phenotype of these combinations was the result of synthetic sterility (Hackstein et al. 1982). These investigators observed several cases where two mutations in different fertility genes, including combinations of a mutation in gene *Q* with mutations in either gene *A* or *C*, failed to complement. This phenomenon was called synthetic sterility. It is thought to reflect defects in cooperative interactions between the two mutated gene products. Therefore, these experiments alone are not sufficient to completely exclude the possibility that there is an additional complementation group on the short arm that is mutable to male sterility.

To circumvent these uncertainties, male-sterile mutations were induced on *T(X;Y)* chromosomes carrying gene *Q* and only a few other fertility genes. Three male-sterile alleles of gene *Q* were X-ray-induced by J.H.P. Hackstein on *T(X;Y)47* (Hess 1965b), carrying fertility genes *N*, *O*, *P* and *Q*, and four male-sterile mutations were induced in either gene *N*, *O* or *P*. These seven mutations all showed a regular complementation with *ms(Y)Q1* to *ms(Y)Q6*, *i.e.* the three mutations in gene *Q* were sterile in combination with any of the *ms(Y)Q* alleles, whereas the four others were fertile. In addition, 27 male-sterile mutations were X-ray-induced by J.H.P. Hackstein on *Df(YL)S0*, carrying fertility genes *O*, *P*, and *Q* (Hackstein and Hennig 1982). Mutations in gene *Q* were recognized by their failure to complement *T(X;Y)S6*, and because they complemented *T(X;Y)B9*, lacking a functional gene *O*. All mutations in gene *Q* induced on *Df(YL)S0* failed to be complemented by *ms(Y)Q1* (J.H.P. Hackstein, personal communication).

Altogether, more than 200 combinations involving sterile alleles on a free *Y* chromosome, sterile alleles on *T(X;Y)*s, and fertile alleles on *T(X;Y)*s were tested for complementation. It is unlikely that the breakpoint in each of the different *T(X;Y)*s is at an identical position in the short arm. Therefore, there is no evidence

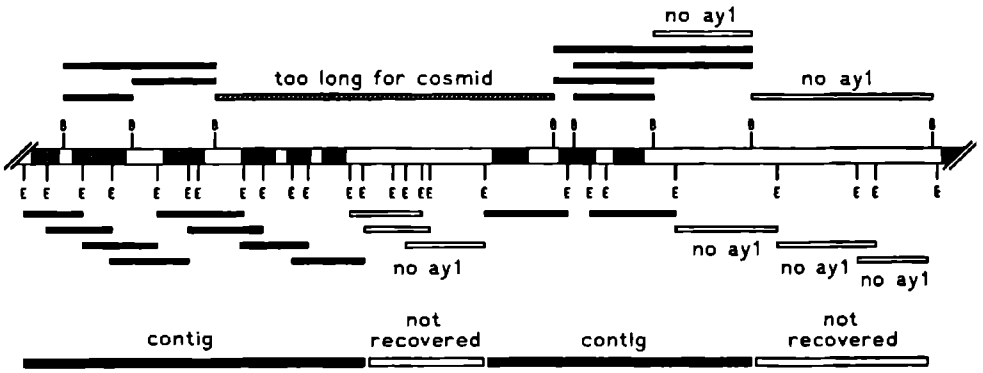


Fig 5. Experimental strategy for the cloning of DNA from the lampbrush loop pair *Nooses*. A hypothetical restriction map of a stretch of DNA from the *Nooses* loop is shown, based on previous work of Vogt and Hennig (1983, 1986a,b). The *Y*-specific ay1 repeats, which are assumed to occur in small clusters of 3-5 repeats throughout the lampbrush loop-forming transcription unit, are indicated by *hatched rectangles*. The ay1 repeat clusters are interspersed by *Y*-associated DNA sequences, which are indicated by *open rectangles*. Restriction sites for BamHI (*B*) and EcoRI (*E*) are indicated. *Thin black bars* above (BamHI libraries) and below (EcoRI libraries) the map represent restriction fragments that can be isolated as lambda and cosmid clones by probes containing ay1 sequences. However, it is unknown whether all DNA from the transcription unit can be isolated following this procedure. Fragments with a size suitable for lambda or cosmid cloning, but without ay1 sequences, will not be recovered. Such fragments are indicated by the *open thin bars* above and below the map. Fragments with ay1 repeats, but without suitable restriction sites, will not be included in the libraries, as, for example, the fragment indicated by the *shaded thin bar* above the restriction map. Therefore, from this particular stretch of DNA, two contigs of overlapping genomic clones can be assembled, which are indicated by the *thick black bars* at the bottom of the figure. DNA segments that would be missing are indicated by the *open thick bars*

for a second male fertility gene on the short arm. The function of the single fertility gene on the short arm is strictly correlated with the cytological expression of the *Nooses* loop pair, the only lampbrush loop pair formed by the short arm (see section 3.2 and also Chapter 4). Thus, with respect to gene *Q*, the genetic data are fully consistent with the one loop-one gene relationship, that is even more firmly established for genes *A*, *B* and *C* on the long arm of the *D. hydei Y* chromosome, where more than 300 combinations *of ms(Y)i* and *T(X;Y)* chromosomes have been tested without revealing any evidence for the presence of more than one fertility gene in each loop-forming locus (Hackstein et al. 1991). Also in the case of the three loop-forming genes on the *Y* chromosome of *D. melanogaster*, no evidence has been presented that disproves the one loop-one gene relationship (section 2.2).

(ii) The lampbrush loop pair formed by gene *Q* has a relatively small size: The *Nooses* loop pair is the smallest loop pair known in *D. hydei*: approximately 260 kb of DNA are transcribed in a single transcription unit (Grond et al. 1983), which is much smaller than the sizes of the transcription units of the other loop pairs (Glätzer and Meyer 1981; de Loos et al. 1984; unpublished observations of R. Suijkerbuijk and W. Hennig).

(iii) The repetitive DNA sequences from the loop are sufficiently heterogeneous to enable genomic walking: The analysis of DNA fragments that hybridized to *Nooses* transcripts had revealed considerable sequence heterogeneity among the *Nooses*-specific ay1 repeats. In addition, also *Y*-associated DNA sequences were known to be transcribed in the loop (Vogt and Hennig 1983, 1986a,b). Despite of the repetitive nature of the transcribed DNA sequences, the sequence heterogeneity within the *Nooses* transcription unit would therefore be sufficient to allow the identification of overlapping genomic clones containing DNA from the loop.

(iv) DNA fragments containing ay1 are likely to originate directly from the loop-forming region: The initial analysis of the genomic organization of the ay1 family had indicated that most, if not all, ay1 repeats are located within the 260 kb long transcription unit (Vogt and Hennig 1986a). Therefore, the screening of genomic libraries with ay1 sequences as a probe would permit the direct identification of DNA that is transcribed in the *Nooses*. Most likely, the loop-forming transcription unit would consist of ay1 and *Y*-associated DNA sequences, that are surrounded by other, nontranscribed DNA sequences.

Using at least two different restriction enzymes for the construction of genomic libraries (Chapter 2), it was expected that the cloning of major parts of the 260 kb of DNA transcribed in the loop would be possible, especially since in addition to lambda vectors, also cosmids would be used as cloning vehicles. However, as explained in Fig. 5, not all the loop DNA might be recovered by this procedure, either because of a lack of ay1 repeats, or because of absence of appropriate restriction sites.

The reconstruction of an entire loop-forming transcription unit in an ordered set of overlapping genomic clones would be an important step forward in understanding the structure and the function of the loop-forming fertility genes. Such a reconstruction will permit to address the following questions.

(i) What is the function of the loop-forming genes? Are they protein coding genes (see section 4.6), with exons that are conserved between the different *Drosophila* species? If so, what is the gene product? Are the exons located within the loop-forming transcription unit?

(ii) Why does fertility gene function require the formation of a giant lampbrush loop? Is loop formation simply the result of a failure to terminate the transcription of an upstream located, protein coding gene?

(iii) What is the structure of the promoter that initiates transcription of the lampbrush loop? Is this structure conserved between the different loops? Are there *trans*-acting factors that activate the loop promoter? Male-sterile mutations on are known that prevent or affect the formation of all lampbrush loop pairs during meiotic prophase (Kiefer 1973; Lifschytz 1974,1975; Hackstein et al. 1987,1990; Hackstein 1991), indicating that loop unfolding is under control of genes located on other chromosomes.

(iv) What is the structure of the terminator of lampbrush loop transcription? Usually, termination in eukaryotic genes that are transcribed by RNA polymerase II is based on specific sequences in the DNA, such as the polyadenylation signal 5' AATAAA 3', which is highly conserved from yeast to mammals (reviewed by Proudfoot 1989; Manley 1990). Loop transcripts detected with *Y*-specific, repetitive DNA sequences as a probe, are most likely not polyadenylated (Lifschytz et al. 1983; Wlaschek et al. 1988). However, are there polyadenylated (m)RNAs that are spliced out from the giant, primary loop transcripts?

(v) Which aspects of the molecular organization within the lampbrush loops are conserved between the different *Drosophila* species that display such an enormous variation of loop morphology? Which processes have contributed to the extremely rapid evolution of the large lampbrush-loop forming genes?

(vi) Is it possible to identify the mutant lesion in male-sterile alleles of the loop-forming genes? With respect to gene *Q*, the temperature-sensitive allele $ms(Y)Q4^{ts}$ (Leoncini 1977) is particularly interesting.

7.2 An outline of this thesis

The work described in this thesis was aimed at reconstructing the lampbrush loop pair *Nooses* in a set of overlapping genomic clones. Due to the unexpected molecular features of the short arm of the *Y* chromosome, such a reconstruction has

not yet been possible. However, considerable progress towards this aim has been made and the principles of the organization of the DNA sequences in the short arm of the *Y* chromosome have been worked out. It was found that the *Y* chromosome contains much more ayl sequences than the 260 kb expected from earlier studies. However, four lambda and at least two cosmid clones were isolated that, as will be in shown the following chapters, have all the properties of being a major part of the *Nooses*.

In **Chapter 2** the strategy for the cloning of the DNA transcribed in the *Nooses* loop pair is discussed in more detail, and an initial characterization of potential DNA segments from the loop is presented. Methods to verify whether the isolated ayl-containing clones indeed represent segments of the loop-forming transcription unit are developed in Chapters 3, 4 and 5. In **Chapter 3**, the different types of repetitive DNA sequences that could be distinguished, are localized at distinct positions on the *Y* chromosome, only one of which coincides with the loop-forming gene. Other criteria for distinguishing between the transcribed and nontranscribed repetitive DNA sequences are given in **Chapter 4** and in **Chapter 5**. It will be shown that the loop DNA does not contain YsI repeats, but mainly consists of ayl repeats that are interspersed by retrotransposons of the *gypsy* family. In **Chapter 6** the sequence analysis of the transcribed ayl repeats is presented, and in **Chapter 7** that of the *gypsy* sequences. The transcription of these major loop constituents in male-sterile alleles of fertility gene *Q* is studied in **Chapter 8**. **In Chapter 9**, these findings are discussed in the context of fertility gene function and evolution. The DNA sequences determined during the course of this work are listed in the **Appendix**.

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CHAPTER 2

Partial reconstruction of the lampbrush loop pair *Nooses* on the *Y* chromosome of *Drosophila hydei*

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Abstract. We present the analysis of genomic DNA fragments that were isolated as potential segments of the lampbrush loop pair *Nooses* on the short arm of the *Y* chromosome of *Drosophila hydei* More than 300 kb of DNA were recovered in BamHI lambda and cosmid clone groups This DNA is composed of the *Y* specific ay1 family of repetitive DNA sequences, and of other repetitive DNA sequences which at least in part are also located elsewhere in the genome (*Y*-associated sequences) Two additional classes of DNA fragments were obtained from an EcoRI library One of them consists of ay1 repeats without apparent interspersion including a total of more than 300 kb of DNA The other is composed of tandemly repeated Ys1 sequences a *Y* specific sequence derived from ay1 This class includes more than 400 kb of DNA which is also not interspersed by other sequences Our results show that only the ay1 repeats interspersed by *Y*-associated DNA sequences can represent parts of the 260 kb transcription unit forming the lampbrush loop, whereas the ay1 and Ys1 repeats without interspersion form separate and nontranscribed clusters of repetitive DNA

Introduction

During the male meiotic prophase several fertility genes on the *Y* chromosome of *Drosophila hydei* form lampbrush loops with lengths of several hundred to more than thousand kilobases (reviewed by Hennig 1985 1990 Hennig et al 1989) These lampbrush loops are large transcription units that are composed of a complex pattern of repetitive DNA sequences Single copy sequences or protein coding sequences have so far not been identified

Earlier work from our laboratory has successively provided molecular evidence for the structure of these loop forming fertility genes, allowing the design of a working model for their general structure (Hennig 1968 1987 Hennig et al 1974 1983 1989, Vogt et al 1982 1986 Vogt and Hennig 1983 1986a, b Huijser and Hennig 1987) According to this model *Y*-specific repeats are interspersed with repetitive DNA sequences of a different structure which also occur on other chromosomes and have therefore been designated as *Y*-associated (Vogt and Hennig 1983 Hennig et al 1989 Hennig 1990) *Y*-associated sequences are transcribed in loops and at least some of them represent transposable elements (Huijser et al 1988)

Our working model is based on hybridization experiments, using cloned repetitive DNA sequences, or their in vitro transcripts as probes However the hybridization of probes of only a few kilobases length to loop transcripts with a size of many hundred kilobases and composed of repetitive sequences does not prove that the particular sequence used as a probe is indeed present within the transcription unit We therefore decided to obtain non-circumstantial evidence for the validity of our model by a systematic reconstruction of one of the fertility genes at the DNA level Our approach includes (i) the isolation of all DNA fragments that are potential parts of the fertility gene and (ii) the alignment of the isolated fragments by searching for overlapping restriction patterns This latter step should be feasible as our prior studies have indicated considerable heterogeneities within the repetitive DNA sequences of *Y*-chromosomal lampbrush loops This should suffice to distinguish different DNA fragments (see for example Hennig et al 1983, Vogt and Hennig 1986a b Huijser et al 1988)

We have focussed on the smallest lampbrush loop pair of the *D hydei Y* chromosome the loop pair

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Nooses It is correlated with the fertility gene *Q* on the short arm of the *Y* chromosome (Hackstein et al 1982) As can be visualized in Miller spreads of transcribed chromatin, the loop is a single transcription unit of not more than 260 kb length (Grond et al 1983) We have shown earlier that the *Y*-specific ay1 family of repetitive DNA sequences is specifically transcribed in this lampbrush loop pair (Vogt et al 1982, Vogt and Hennig 1986a) and also that *Y*-associated sequences are transcribed in the loop (Vogt and Hennig 1986b) Other investigators have claimed that in addition to ay1 repeats, repeats of the *Y*-specific YsI family are also transcribed in the *Nooses* (Lifschytz et al 1983, Trapitz et al 1992) YsI is an evolutionary derivative of the more ancient ay1 family (Vogt and Hennig 1986a), and repeats of both families cross-hybridize (Wlaschek et al 1988)

In this paper we report the recovery of potential segments of the *Nooses*, using ay1 repeats to screen libraries of genomic DNA Three classes of clones were recovered 300 kb of clones containing ay1 repeats that are interspersed by unrelated, so-called ay1-associated sequences, another 300 kb of clones containing ay1 repeats without apparent interspersion, and 400 kb of clones containing uninterrupted repeats of the YsI family Together, these clones represent considerably more DNA than can be accommodated within the 260 kb loop length However, several of the *Y*-associated sequences, which interrupt ay1 repeats, are specifically transcribed in the *Nooses* Further, we show in the accompanying paper (Hochstenbach et al 1993), that interspersed ay1 repeats and homogeneous clusters of ay1 and YsI are located in separate domains on the short arm of the *Y* chromosome We therefore conclude that only clones containing ay1 repeats and sequences of the *Y*-associated type represent segments of the transcription unit

Materials and methods

Fly strains *D hydei* individuals were taken from the Tubingen wild-type strain from our laboratory collection The *D hydei* wild-type strains Zurich Alicante and Madeira were kindly provided by Dr F M A van Breugel (University of Leiden) Males lacking the short arm of the *Y* chromosome, and therefore lacking fertility gene *Q* and the associated loop pair *Nooses*, were generated by crossing virgin wild-type females to T(*X*,*Y*)59/GE7 males GE7 is a *Y* chromosome without the short arm, which is complemented by T(*X*,*Y*)59 a translocation of the short arm of the *Y* chromosome to the euchromatic arm of an *X* chromosome carrying the markers yellow, miniature and cherry (Hackstein et al 1982, Hackstein and Hennig 1982) Absence of the short arm was confirmed by inspection of brain metaphases of *X*/GE7 male larvae and by the failure to detect ay1 repeats on Southern blots of genomic DNA prepared from such males (data not shown) Flies were kept at 18° or 24° C on a medium containing dried yeast, cornmeal, soy flour, malt and sugar-beet syrup that was inoculated with live yeast

Preparation of embryonic nuclei Genomic DNA of *D hydei* was isolated from purified embryonic nuclei Eggs were collected overnight in a large population cage on yeasted syrup-agar dishes, washed with tap water and stored at −70° C For isolation of nuclei, 3 5 g thawed eggs were washed repeatedly by settling in 50 ml ice-cold saline [0 9% (w v) NaCl, 0 1% (v v) Triton X-100], and dechorionated in 20 ml commercial bleach (containing sodium hypochlorite at a final concentration of 3%) The dechorionated embryos were collected by filtration through nylon mesh transferred to a 30 ml conical centrifuge tube washed with distilled water and sedimented at 4° C by centrifugation at 1500 rpm in a Sorvall HB-4 rotor Embryos were resuspended washed and centrifuged repeatedly until a clear supernatant was obtained Finally, the embryos were resuspended in 10 ml ice-cold Buffer I [10 mM Tris HCl pH 7 0 1 5 mM $MgCl_2$, 0 3 mM $CaCl_2$, 0 5 mM DTT (dithiothreitol) 2% (w/v) dextran-500]

Embryos were manually homogenized and the homogenate was filtered through Miracloth (Calbiochem) Nuclei were collected by centrifugation at 4° C in an HB 4 rotor for 15 min at 2100 rpm Nuclei were resuspended in fresh Buffer I, washed and sedimented again This procedure was repeated once more and the final pellet was resuspended in Buffer II (100 mM Tris-HCl, pH 8 2 100 mM EDTA)

Preparation of genomic DNA Prewarmed aliquots of 50 µl nuclei were mixed with an equal volume of 2 0% (w v) low melting agarose (BioRad) in Buffer II at 50° C, and allowed to solidify on ice in a plastic mould The agarose blocks thus obtained were equilibrated twice for 1 h each at 50° C in an excess volume of proteinase K buffer [10 mM Tris HCl pH 7 5, 250 mM EDTA, 1% (w v) sarkosyl 0 2% (w v) sodium desoxycholate] and then treated overnight in the same buffer with 0 1% (w/v) self digested proteinase K at 50° C Proteinase K was inactivated by immersing the blocks in 1 mM PMSF (phenylmethylsulphonyl Fluoride) in TE buffer (10 mM Tris-HCl pH 8 0, 1 mM EDTA), followed by washing the blocks three times for 1 h each at 37° C The agarose blocks were subsequently washed in excess volumes of RNase buffer [10 mM Tris-HCl pH 7 5 100 mM EDTA, 1 M NaCl 0 5% (w v) sarkosyl, 0 2% (w/v) sodium desoxycholate] and incubated in the same buffer with pre-boiled RNase A at 200 µg ml at 37° C overnight

For partial digestion with BamHI or EcoRI, agarose blocks were equilibrated three times for 1 h each in excess volumes of 1 × Buffer B or H (Boehringer Mannheim) respectively For cleavage of genomic DNA blocks were placed in 0 5 ml restriction buffer supplemented with 0 1 mg ml BSA, 2 mM spermidine and 1 mM DTT and incubated overnight at 37° C The amount of enzyme was adjusted to yield genomic DNA fragments in the 20-40 kb range Fragment length was controlled using an LKB Pharmacia 2015 Pulsaphor pulsed-field electrophoresis unit To prevent ligation events between genomic DNA fragments blocks were first equilibrated in 50 mM Tris-HCl, pH 8 0 0 1 mM EDTA three times for 1 h each at 37° C and then incubated in the same buffer with 2 units CIP (calf intestinal phosphatase Boehringer Mannheim) for 2 h at 37° C CIP was inactivated by immersing the inserts in TE containing 0 1% (w/v) SDS

Agarose blocks were melted in 300 µl TE at 65° C the solution was carefully extracted with phenol, extracted again and DNA fragments were fractionated on a 15 ml 10%-40% (w/v) sucrose gradient in 10 mM Tris-HCl, pH 8 0, 1 M NaCl, 10 mM EDTA by centrifugation overnight at 18° C in a UCB-SB283 rotor at 28000 rpm For lambda libraries, 10 2·3 kb fractions were pooled, and for cosmid libraries 25 40 kb fractions Pooled DNA fragments were precipitated and dissolved in TE

Construction of the cosmid library The pHC79 cosmid vector (Hohn and Collins 1980) was linearized with either PstI or SalI, dephosphorylated using CIP as described above and then cleaved with BamHI The 5 4 kb PstI-BamHI and 6 2 kb BamHI-SalI vector arms were isolated from a low melting agarose gel and purified About 1 µg of genomic DNA fragments (25–40 kb) was mixed with 0 4 µg of each vector arm and ligated overnight at 16° C in a volume of 30 µl containing 50 mM Tris-HCl pH 7 6 10 mM $MgCl_2$, 50 µg ml BSA 1 mM ATP and 1–1 5 unit T4 ligase For in vitro packaging, 7 5 µl of the ligation mix was incubated with 25 µl packaging extract (Promega Packagene System) for 2 h at 22° C, after which 0 5 ml SM buffer [50 mM Tris-HCl, pH 7 5, 100 mM NaCl, 8 mM $MgCl_2$ 0 01% (w/v) gelatin] and 20 µl chloroform was add

ed Recombinants were transduced in *Escherichia coli* HB101 cells [grown to log phase in LB medium supplemented with 10 mM $MgCl_2$ and 0.2% (w v) maltose] following standard procedures (Sambrook et al 1989) and cells were plated out on LB agar plates containing 50 μg ml ampicillin An efficiency of 3.5×10^5 colonies per microgram packaged DNA was obtained

Construction of lambda libraries Lambda libraries were constructed in λEMBL3 or λEMBL4 (Frischauf et al 1983) A library of genomic DNA from males containing BamHI fragments in λEMBL3 was constructed by Vogt and Hennig (1986a) An additional EcoRI library was constructed in λEMBL4 One microgram vector DNA was digested to completion with BamHI and EcoRI and mixed with 1.5 μg of fractionated 10-23 kb EcoRI fragments Ligation and packaging were as described above Recombinant phages were transduced in *E coli* Q358 (Karn et al 1980) or NM538 cells (Frischauf et al 1983) following standard procedures (Sambrook et al 1989) Efficiencies of $1.2-3 \times 10^6$ pfu per microgram packaged DNA were obtained Libraries were amplified by plating 160000-210000 pfu

Screening of genomic libraries The BamHI pHC79 cosmid library was plated on 22 × 22 cm dishes at a density of about 10000 recombinants per dish Altogether 145000 recombinants were screened (containing about 11 male genome equivalents see Table 1) About 115000 plaques from the BamHI λEMBL3 library (corresponding to 4 male genome equivalents) and 150000 plaques from the EcoRI λEMBL4 library (corresponding to 5 male genome equivalents) were screened (see Table 1) Recombinants were immobilized on Hybond N membranes (Amersham) and membranes were processed following instructions from the manufacturer Clones containing ay1 were identified by hybridization with insert from plasmid clone PY9 (Vogt et al 1982) that was labelled with ^{32}P by nick translation (Sambrook et al 1989) PY9 insert was purified from agarose gels according to the freeze squeeze method of Tautz and Renz (1983) PY9 contains a 9.0 kb PstI DNA fragment that consists of about 20 tandemly arranged ay1 repeats PY9 or a single ay1 repeat give identical hybridization patterns on genomic DNA from males (Vogt and Hennig 1986a) Hybridization was in 2 × SSC 0.1% (w v) SDS at 58° C (1 × SSC is 0.15 M NaCl 0.015 M sodium citrate pH 7.2) Filters were washed in the same solution at room temperature and exposed at −70° C to Kodak X-Omat film using Dupont Cronex intensifying screens

Analysis of recombinants Cosmid DNA was isolated from saturated 5 ml cultures according to the alkaline lysis method of Birnboim and Doly (1979) Lambda DNA was isolated from 10 or 40 ml cultures following the procedure of Dumanski et al (1988) Restriction fragments were separated on 0.8%–1.2% agarose gels immobilized on Hybond N membranes and probed with nick translation labelled PY9 insert to identify restriction fragments harbouring ay1 repeats Initial washing of the membranes was in 2 × SSC 0.1% (w v) SDS at 65° C allowing PY9 to hybridize both to ay1 and YsI sequences To distinguish fragments containing ay1 from those containing YsI membranes were subsequently washed in 0.1 × SSC 0.1% (w v) SDS at 65° C Under these conditions PY9 will hybridize only weakly to YsI sequences (see Figs 2 and 3) For subcloning selected restriction fragments were purified from agarose gels and cloned in pGEM3 (Promega) or pBluescriptII KS (Stratagene) plasmid vectors following standard protocols (Sambrook et al 1989) DNA of pGEM3 plasmids was isolated according to Birnboim and Doly (1979) that of pBluescriptII KS plasmids according to the boiling procedure recommended by Stratagene

Southern hybridization of genomic DNA Genomic DNA was isolated from adults as described by Huijser and Hennig (1987) enzymatically cleaved and separated on 0.45% 1% agarose gels DNA was transferred to Hybond N membranes and hybridized with nick translation labelled gel purified restriction fragments under conditions specified by the manufacturer

Nonradioactive in situ hybridization For in situ hybridizations to polytene chromosomes squashes of third instar larval salivary glands were prepared RNase treated and denatured as described (Hennig et al 1982)

For transcript in situ hybridization testis of young adult males were dissected in a buffer containing 183 mM KCl 47 mM NaCl 10 mM Tris HCl pH 6.8 and gently squashed using a siliconized coverslip Slides were frozen in liquid nitrogen and after removal of the coverslip stored in absolute ethanol at 4° C The tissue was then treated following a modification of the protocol of Tautz and Pfeifle (1989) Slides were air dried and the tissue was fixed for 20 min at room temperature (RT) in 1.0% (v v) formaldehyde in PBS (3 mM KCl 1.5 mM KH_2PO_4 137 mM NaCl 7 mM Na_2HPO_4 with pH adjusted to 7.4) All washing steps of the slides were for 5 min at RT First slides were washed three times in PTw [PBS containing 0.1% (v v) Tween 20] and incubated in 20 μg ml pre-digested proteinase K in PTw for 1 min After washing two times in PTw containing 2 mg/ml glycine and three times in PTw the tissue was fixed again in 3.7% (w v) paraformaldehyde in PBS for 20 min at RT

For prehybridization of both salivary gland squashes and testis squashes 20 μl of hybridization buffer [HB 50% (v v) formamide 5 × SSC 50 μg ml *E coli* tRNA 50 μg ml heparin 0.1% (v v) Tween 20] was applied on the tissue and sealed with a coverslip Slides were incubated for 1 h at 50° C in a moist box For hybridization 10 μl of fresh HB containing about 20 ng probe RNA was applied the coverslip was sealed with rubber cement and slides were incubated overnight at 50° C The RNA probes containing digoxigenin-11-UTP (Boehringer) were in vitro transcribed from linearized pBluescriptII KS plasmids using either T3 or T7 RNA polymerase following Boehringer Mannheim protocols

After hybridization slides were successively washed in the following solutions 3:1 HB 2 × SSC 1:1 HB 2 × SSC 1:3 HB 2 × SSC and 2 × SSC Non specifically bound probe RNA was removed by incubating slides in 10 μg ml RNase A in 2 × SSC for 30 min at 30° C Slides were then washed in 0.2 × SSC 1:3 PTw 0.2 × SSC 1:1 PTw 0.2 × SSC 3:1 PTw 0.2 × SSC and PTw To prevent non-specific binding of the anti-digoxigenin antibody slides were incubated in PBT [PBS containing 2 mg ml BSA 0.1% (v v) Triton X 100] for 15 min at RT followed by incubation in PBT containing 0.5% (w v) non fat milk powder for 1 h at RT Subsequently slides were incubated for 1 h at RT in the same solution containing 1:100 diluted anti digoxigenin sheep Fab fragments conjugated with alkaline phosphatase (Boehringer Mannheim) followed by three washes in PBT

The position of probe RNA was visualized by washing slides three times in NBT buffer [0.1 M Tris HCl pH 9.5 0.05 M $MgCl_2$ 0.1 M NaCl 0.1% (v v) Tween 20] followed by developing slides in NBT buffer containing 5 bromo 4 chloro 3 indolyl phosphate (Serva) at 37.5 μg ml and 4 nitroblue tetrazolium chloride (Serva) at 20 μg ml at RT until a coloured precipitate was formed Photographs were taken under phase contrast optics on Agfapan 100 professional film

Results

The strategy for mapping the Nooses loop and its underlying assumptions

Our approach to reconstruct the DNA of the entire *Nooses* lampbrush loop in a set of overlapping clones was encouraged by the assumption that the amount of repetitive DNA transcribed in the loop is about 260 kb, as shown by Miller spreading experiments (Grond et al 1983) To isolate these clones we adopted the following three-step strategy

l. Summary of all PY9-hybridizing clone groups from four different different libraries

y	Vector	Enzyme	Genome equivalents screened[a]	Clones	Groups	Insert sizes (kb)	Total length (kb)[b]	Cumulative amounts (kb) of	
								total DNA[c]	ay
d	pBR322	PstI	0.5	4[e]	4	2.2–10.5	25.0	25.0	
la	λEMBL3	BamHI	4	42	12	11–20	217.7	180.2	
d	pHC79	BamHI	11	8	5	33–39	180.1	130.3	
la	λEMBL4	EcoRI	5	108[f]	58	11–20	894.4	740.5	
								∑ 1055.9[g]	∑

umber of genome equivalents screened for each library was ted on the basis of the number of screened recombinants, verage insert length, and the size of the diploid male genome *sophila hydei*, which is about 460000 kb (Zacharias et al.

numbers represent the cumulative length of all clone groups d from each library without correction for overlaps between nt groups

numbers are the cumulative lengths of all different clone isolated from a particular library after correction for over- or the BamHI clones only the BamHI fragments with ay1 shown in Fig. 4 are taken into account

[d] These numbers are the cumulative lengths of all different tion fragments hybridizing to PY9 from the different clone isolated from each library; they certainly represent a ma value

[e] See Vogt and Hennig (1983), and Vogt and Hennig (19 for a detailed description of these clones

[f] Initially, more than 800 clones were isolated, from whi were randomly chosen for further analysis

[g] These numbers represent the cumulative length of all clone after correction for overlaps between the different groups

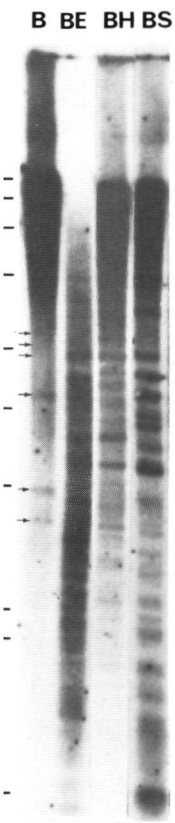


Fig. 1. Southern blot of genomic DNA from adult males hybridized with the ay1-containing probe PY9. DNA fragments were separated on a 0.45% agarose gel for better visualization of large fragments. Hybridization with ^{32}P nick translation-labelled PY9 insert was in 6 × SSC at 65° C; post-hybridization washes were in 2 × SSC at 65° C. These conditions permit cross-hybridization between repeats of the ay1 and YsI repeat families (see Figs. 2 and 3). The bulk of the genomic DNA containing ay1 repeats is infrequently cleaved by BamHI (lane *B*), but some small fragments are seen at 3.8, 4.4, 7.0 and 10–11 kb (indicated by the *small arrows*). Addition of either EcoRI (lane *BE*) or SalI (lane *BS*) results in many small fragments that hybridize to PY9. Thus, these enzymes cleave much more frequently in ay1-containing DNA compared with BamHI. HindIII cleaves less frequently than either EcoRI or SalI, although small fragments are seen (lane *BH*)

1. Screening BamHI libraries of genomic DNA fo segments using ay1 repeats as a probe. The repe is specifically transcribed in the *Nooses*. Because a peats do not hybridize to DNA of females, and are sively located on the short arm of the *Y* chrom (Vogt and Hennig 1983), all isolated genomic must originate from the short arm. The choice of B lambda and cosmid libraries was based on our ob tion that in BamHI digests of genomic DNA from a considerable proportion of the fragments with a peats have sizes that can be cloned in lambda or c vectors (Fig. 1). BamHI recognition sites are rare quenced ay1 and YsI repeats (Vogt and Hennig 1 b; Lifschytz 1987; Wlaschek et al. 1988; R. Hoc bach, M. Knops, H. Harhangi and W. Hennig, u lished data), and therefore we assume that rec fragments contain additional sequences of the *Y* ciated type, which form a substantial part of the scription unit (Vogt and Hennig 1986b). The cor mapping of the loop will be facilitated by ident the differences in size and sequence composition different ay1 clusters within the loop, as well as th ferent types and lengths of ay1-associated sequenc tween the ay1 clusters (Hennig et al. 1989; Hennig

2. Creating restriction maps of the recovered clon assigning them to different groups of identical inser ments. Step (1) will yield a large number of clone all contain ay1 repeats. These clones can be distingu by constructing their restriction maps using EcoRI dIII and SalI. As can be seen in Fig. 1, EcoRI an and to a lesser extent HindIII, frequently cleave B fragments that contain ay1 repeats. SalI is partic useful because frequent cleavage by this enzyme is nostic for repeats of the YsI family (Fig. 2; see also chytz 1987; Wlaschek et al. 1988). In this way ever

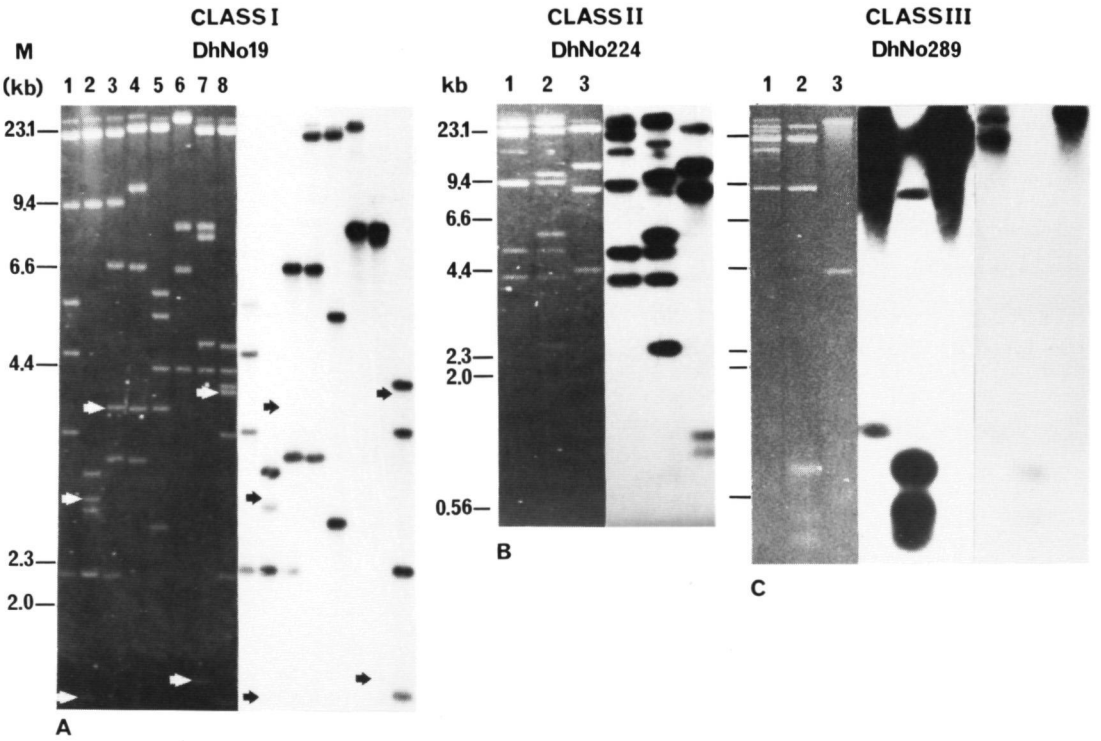


Fig. 2A–C. Classification of ay1-containing clone groups. Restriction fragments of each clone were separated on 0.8%–1.2% agarose gels, stained with ethidium bromide (left panels in **A**, **B** and **C**), immobilized on nylon membranes and hybridized with ^{32}P nick translation-labelled PY9 insert. The right panels in **A** and **B** show corresponding autoradiographs after washing under stringent conditions (0.1 × SSC, 65° C), and the central and right panels in **C** show the corresponding autoradiographs after washing under non-stringent (2 × SSC, 65° C), and stringent conditions, respectively. As a first step, clones with similar inserts were assigned to a group. In a second step, the groups could be assigned to one of the following three classes by their restriction pattern and hybridization pattern to PY9: **A** Class I contains clone groups with restriction fragments that did not hybridize to PY9. Shown here is clone DhNo19, defining group DhNo19. Fragments without ay1 sequences are indicated by *white arrows* in the gel, and their corresponding positions in the autoradiograph are indicated by *black arrows*. All restriction fragments hybridizing to PY9 contain ay1 sequences because the signals remain after washing at high stringency. Restriction enzymes were SalI (lane 1), EcoRI + SalI (lane 2), BamHI + EcoRI (lane 3), EcoRI (lane 4), EcoRI + HindIII (lane 5), HindIII (lane 6), BamHI + HindIII (lane 7) and HindIII + SalI (lane 8). **B** Class II contains clone groups with all restriction fragments hybridizing to PY9 after washing under stringent conditions. Shown here is clone DhNo224 from group DhNo224. Restriction enzymes were BamHI + EcoRI (lane 1), EcoRI + SalI (lane 2) and HindIII (lane 3). **C** Class III contains clone groups with all restriction fragments hybridizing to PY9 but only under non-stringent conditions (central panel). In addition, all groups of Class III clones were found to display a characteristic pattern of small SalI fragments (see Fig. 5). Shown here is clone DhNo312 from group DhNo289, with SalI fragments of about 0.8, 0.4 and 0.25 kb length (see Table 4A). These fragments contain YsI sequences since they fail to hybridize to PY9 after washing under stringtent conditions (right panel). The autoradiograph at the right was exposed twice as long as that in the middle. Restriction enzymes were as in **B**

gle clone can be assigned to a group consisting of clones with identical inserts. If sufficient internal BamHI sites are found, an alignment of the different groups of clones into overlapping contigs might become possible simply on the basis of the restriction maps.

3. Completing loop mapping by screening genomic libraries made with different enzymes for overlapping clones. Screens of at least one library constructed with another restriction enzyme will yield clones that overlap with the BamHI clones. Overlapping parts can be recognized by restriction fragment patterns identical to the outer parts of a particular group of BamHI clones. Such overlapping DNA fragments can be isolated in two ways. Firstly, ay1 can be used as a probe to screen other libraries. This approach has the advantage that only clones of *Y*-chromosomal origin are recovered. Alternatively, fragments with ay1-associated sequences from the outer ends of the BamHI clones can be used for the direct screening for overlapping genomic fragments. A disadvantage of this approach is that the isolated fragments are not necessarily of *Y*-chromosomal origin since the particular fragment used as a probe may have additional copies on other chromosomes, as has been demonstrated for the *Y*-associated sequence of MY3 (Vogt and Hennig 1986b).

This strategy for the complete reconstruction of the lampbrush loop depends on the assumption that no long DNA sections exist in the loop without ay1 sequences, or without homologies to other, interspersed DNA sequences. In the present paper, we will investigate the validity of these assumptions. In addition, we will test whether an EcoRI genomic library yields clones that overlap with BamHI clones, and for several ay1-associated sequences we will test whether they are suitable probes for this purpose.

Nomenclature and classification of cloned DNA fragments

Using purified PY9 insert containing nothing but repeats of the ay1 family (Vogt et al. 1982) as a probe, we screened the three different genomic libraries. Clones from the BamHI cosmid library were designated as DhNocos1, etc. Clones from the BamHI lambda library received the numbers DhNo1 to DhNo199 and those from the EcoRI lambda library started with the number DhNo200. After rescreening of the inital isolates from the BamHI libraries, the remaining 8 cosmid and 42 lambda clones were subjected to further analysis by restriction mapping. From the EcoRI library more than 800 clones were initially isolated. After rescreening of 205 randomly chosen clones, 108 clones were eventually used for restriction mapping (Table 1).

In several cases, we found that different clones from one library contained the same genomic fragment as judged from their patterns of restriction fragments. All clones with identical inserts were assigned to one group, each group thus having one or several members. In the case of a saturating screen, each group should be represented by several identical clones. They are assumed to represent the same genomic DNA sequence as we have no evidence in favour of the occurrence of long identical stretches of DNA. Together, all clones contained about 1300 kb of DNA, with a total sequence heterogeneity of more than 1000 kb (Table 1). This latter number represents about four times the size of the *Nooses* lampbrush loops, and therefore we expected that a major part of the loop would be represented by these clones.

On the basis of the restriction fragments and the hybridization patterns of the different genomic inserts, all groups could be assigned to one of the following three classes (Fig. 2). Class I consists of DNA inserts with some restriction fragments hybridizing to PY9, even under stringent washing conditions, and of other fragments that do not hybridize to PY9. These restriction fragments must therefore contain ay1-associated sequences (Fig. 2A; Table 2). Classes II and III consist of DNA inserts with all of their restriction fragments hybridizing to PY9. Class II does not have the multiple SalI sites typical of the YsI family, and all restriction fragments hybridize to PY9 under stringent conditions (Fig. 2B, Table 3). Class II clones therefore contain repeats of the ay1 family. Class III is characterized by the presence of many SalI sites. All restriction fragments hybridize to PY9, but only under non-stringent conditions (Fig. 2C, Table 4). Therefore, Class III clones contain repeats of the YsI family (Wlaschek et al. 1988; also see Lifschytz 1987). Repeats of the ay1 and YsI families share about 75% sequence homology, while repeats of the same family share about 84% homology (Vogt and Hennig 1986a, b; Wlaschek et al. 1988; R. Hochstenbach, M. Knops and W. Hennig, unpublished data). Therefore, repeats of both families can be easily distinguished using stringent hybridization conditions (Fig. 3).

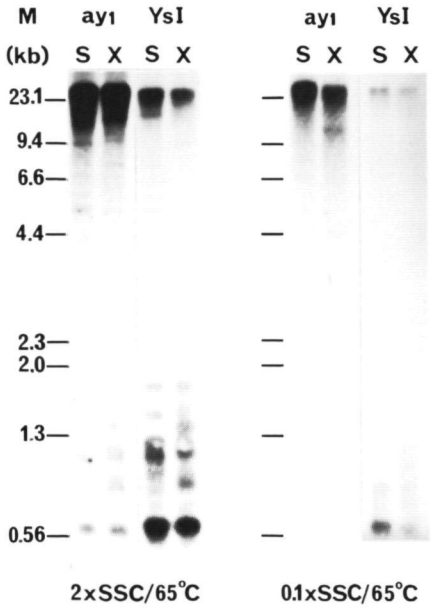


Fig. 3. Southern blots of genomic DNA from adult males, hybridized with the original 393 bp ay1 repeat as defined by Vogt and Hennig (1986a) and a 562 bp YsI sequence from Class III clone DhNo255. Genomic DNA was cleaved by SalI (*S*) or XbaI (*X*), and separated on a 0.8% agarose gel. Two identical membranes were hybridized with ^{32}P nick translation-labelled gel-purified insert of either the ay1- or YsI-specific probe, washed at low stringency as indicated, exposed to X-ray film, then washed at high stringency and exposed again. Each probe detects sequences from the other family at low stringency (left panel; exposure 6 days with two intensifying screens; after longer exposures, both probes hybridize to the same DNA fragments, albeit at somewhat different intensities). After washing at high stringency (right panel; exposure 14 days with two intensifying screens), the ay1 probe reacts mainly to large DNA fragments that are not resolved in the gel, whereas the YsI probe mainly reacts to fragments of about 0.55–0.6 kb

From several Class I clones, DNA fragments containing ay1-associated DNA were subcloned for further analysis. The names of these DNA fragments include the name of the clone from which they originate, the restriction sites at their ends, and their length. For example, DhNo90BE5.8 refers to the 5.8 kb BamHI – EcoRI fragment from clone DhNo90.

Below we will describe the results of our analyses of clones from these three classes, and their implications for the reconstruction of the *Nooses*.

Analysis of Class I clones proves that the Y chromosome contains more DNA with interspersed ay1 repeats than predicted by the 260 kb Nooses loop length

All clones recovered from the BamHI lambda and BamHI cosmid library (Table 2) are heterogeneous and belong to Class I, i.e they contain ay1 as well as ay1-associated DNA sequences, as exemplified by group DhNo19 in Fig 2A The only possible exception is group DhNo5, all restriction fragments of which hybridized to PY9 This does not exclude minor interruptions that cannot be seen in the fragments generated by the particular restriction enzymes used for this analysis. Irrespective of this, clones of Classes II and III containing only ay1 or YsI repeats, respectively, are rare in the BamHI libraries

Of the 12 groups of lambda clones, 8 were isolated only once, and therefore our screen was far from saturation Of these eight groups, three are composed of two different BamHI fragments, only one of which hybridized to PY9 Since we cannot exclude the possibility that these three clones are ligation artefacts, we only show the restriction maps of the ay1-containing BamHI fragments (Fig 4) Groups DhNo55 and DhNo87 were isolated at disproportionate frequencies (Table 2) Since in almost all isolates the insert had the same orientation relative to the λEMBL3 vector, we assume that this reflects a preferential amplification of these groups during bacterial propagation, rather than genomic abundance

From the BamHI cosmid library only five clone groups were isolated and here again, saturation is far from being complete (Table 2) The DhNocos7 and DhNocos18 groups represent genuine sections of genomic DNA. DhNocos7 contains two different BamHI fragments, both of which contain ay1 Group DhNo278 from the EcoRI library contains a piece of DhNocos7 around the internal BamHI site, demonstrating the contiguity of DhNocos7 in genomic DNA DhNocos18 contains only one large BamHI fragment (Fig. 4). The other three groups contain BamHI fragments without ay1 repeats, and do not necessarily represent genuine genomic situations

The relative amounts of ay1-associated DNA can vary widely between the different groups of clones DhNocos7, for example, has a length of 33 4 kb, but less than 10% of it consists of ay1-associated DNA In contrast, DhNocos18, with a length of 38 8 kb, contains at least 63% of ay1-associated DNA. The longest potentially uninterrupted stretch of ay1-associated DNA is found in DhNocos18, and has a minimum length of 17 kb If both DhNocos7 and DhNocos18 were a part of the lampbrush loop, the local density of ay1 sequences

Table 2. PY9-hybridizing clone groups from the BamHI lambda and cosmid libaries

Group	Number of members	Insert length (kb)	BamHI fragments (kb)[a]	Contribution (kb) to	
				total DNA[b]	ay1 DNA[c]
DhNo86	2	11 0	11 0*	11 0	3 5
DhNo90	2	13 5	13 5*	13 5	7 3
DhNo55	15	16 4	12 9*, 3 5	12 9	5 0
DhNo19	1	17 0	17 0*	17 0	8 6
DhNo87	15	17 2	17 2*	17 2[d]	11 1
DhNo16	1	17 2	12 9*, 4 3	12 9	10 0
DhNo17	1	17 2	13 0*, 4 2*	17 2	8 8
DhNo31	1	17 5	13 1, 4 4*	4 4	1 9
DhNo3	1	17 5	16 7*, 0 8	16 7	4 3
DhNo32	1	18 3	18 3*	18 3	16 7
DhNo52	1	18 6	18 6*	18 6	12 3
DhNo5	1	20 5	20 5*	20 5	20 5
				∑ 180 2	∑ 110 0
DhNocos7	1	33 4	27 2*, 7 2*	33 4	30 8
DhNocos47	1	35 1	17 2*, 13 2, 4 7	17 2[d]	11 1
DhNocos18	4	36 2	36 2*	36 2	14 3
DhNocos40	1	36 6	21 3*, 15 3	21 3	21 0
DhNocos6	1	38 8	22 2*, 16 6[e]	22 2	18 1
				∑ 130 3	∑ 95 3
				∑ 293 3[f]	∑ 194 2[f]

[a] BamHI fragments that contain ay1 are indicated by an asterisk (*)

[b] The minimal contribution to the total length of ay1-containing DNA is represented by the ay1-containing BamHI fragments only

[c] This number represents a maximum value based on the length of all ay1-containing restriction fragments present in each group

[d] The ay1-containing BamHI fragment the DhNocos47 group is identical to the BamHI insert of the DhNo87 group

[e] By more detailed restriction mapping and partial sequencing, the 16 6 kb BamHI fragment from group DhNocos6 without ay1 repeats was found to contain a complete rDNA repeat with a 6 kb-long intervening sequence (IVS) in the 28S RNA gene

[f] Including correction for the insert of the DhNo87 group that is also present in the DhNocos47 group

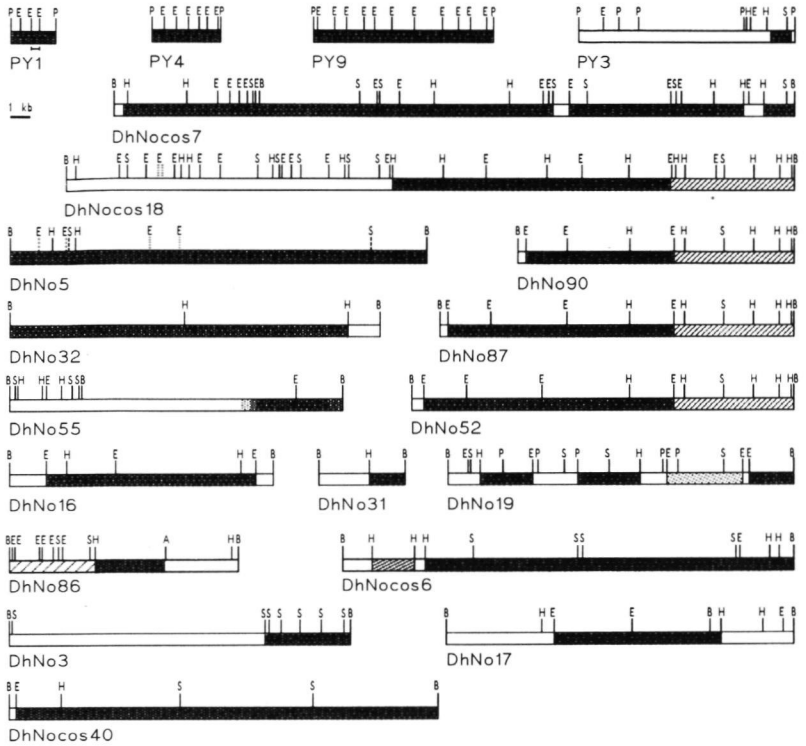


Fig. 4. Restriction maps of 4 plasmid, 12 lambda and 4 cosmid clone groups. The plasmid clones PY1, PY3, PY4 and PY9 were described earlier by Vogt and Hennig (1983). The basic ay1 repeat unit is represented by a 393 bp EcoRI fragment of clone PY1 (Vogt and Hennig 1986a) and is indicated beneath the map of PY1. In PY3, the rightmost PstI fragment (with a size of 2.5 kb) corresponds to MY3 (Vogt and Hennig 1986b). Only the ay1-containing BamHI fragments of the Class I clones are shown. The map of DhNo19 differs from that published earlier (Hennig et al. 1989) as sequence analysis has revealed more details of the composition of this clone (R. Hochstenbach, H. Harhangi, K. Schouren and W. Hennig, in preparation). Restriction fragments that hybridized to PY9 under stringent conditions (0.1 × SSC, 65° C), and hence contain ay1 repeats, are represented by *dark shading*. In cone group DhNo55, the location of ay1 repeats could not be mapped accurately, but for simplicity it is assumed that the repeats in the large BamHI–EcoRI fragment flank those in the smaller fragment, and further that both fragments contain an equal length of ay1 sequences because they have comparable hybridization intensities to PY9 probes. ay1-associated fragments that cross-hybridized under stringent washing conditions (0.1 × SSC, 65° C) are indicated by *identical shading*. Fragments that cross-hybridized only under conditions of low stringency are indicated by *different types of shading*. Restriction enzyme abbreviations are *A* AvaI, *B* BamHI, *E* EcoRI, *H* HindIII, *P* PstI, *S* SalI. Some restriction sites in clone groups DhNo5 and DhNocos18 were assigned to one of two possible positions that could not be distinguished. The two alternative positions are indicated by *identical interrupted* or *dotted lines*

would vary drastically within the loop, and the distances separating two adjacent clusters of ay1 repeats could be as long as 17 kb. Taking all BamHI clones together, the ratio between ay1 and ay1-associated DNA is about 2 to 1. Although restriction sites are not expected to define the boundaries of clusters of ay1 repeats, the average ay1 cluster size of 8.5 kb in the BamHI clones is large compared with the 1 kb cluster size in clone MY3 (Vogt and Hennig 1986b).

In conclusion, from cosmid and lambda BamHI libraries we have recovered 17 different clones, containing BamHI fragments varying in length from 4.2 to 38.8 kb. With the possible exception of DhNo5, all clones belong to Class I, containing ay1 and other, unrelated sequences. Thus, screening genomic libraries constructed with BamHI efficiently selects for clones containing interspersed ay1 repeats. Together with clone PY3 (Vogt and Hennig 1983) these clones represent more than 300 kb of DNA from the short arm of the *Y* chromosome.

Overlapping clones are inefficiently isolated from a genomic library constructed with EcoRI

As a first effort to isolate clones that extend or connect the BamHI clones, we used PY9 to screen a genomic lambda library constructed of partially digested genomic EcoRI fragments. This enzyme was chosen because several of the BamHI clones have small ay1-containing BamHI – EcoRI fragments at their outer ends. HindIII and SalI are less suitable. In combination with BamHI,

Table 3. PY9-hybridizing clone groups from the EcoRI-λEMBL4 library without SalI repeats

Group	Members	Insert (kb)	BamHI sites	HindIII sites	EcoRI fragments (kb)	Fragments from EcoRI + SalI digest (kb)
A Groups with an internal BamHI site						
DhNo278	1	14 6	1	2	6 1, 6 (= 5 8 + 0 2BE), 1 1, 0 6	6 1, 5 2 0 9, 0 8, 0 3
DhNo240	1	15 3	1	1	8 0, 5 9 (= 5 7 + 0 2BE) 1 4	8 0 5 9, 1 4
DhNo201	>4	18 0	1	0	18 0 (= 11 0 + 7 0BE)	18 0
		∑ 47 9				
B Groups without internal BamHII, HindIII and SalI sites						
DhNo326	3	11 9	0	0	3 3, 2 9, 1 5 1 3, 1 1, 1 0, 0 8	3 3, 2 9, 1 5, 1 3, 1 1, 1 0, 0 8
DhNo308	3	12 5	0	0	12 5	12 5
DhNo258	3	13 5	0	0	13 5	13 5
DhNo324[a]	1	13 5	0	0	9 2 2 7 1 0 0 6	9 2 2 7 1 0 0 6
DhNo367	1	11 9	0	0	9 2 2 7	9 2 2 7
DhNo350	1	15 5	0	0	15 5	15 5
DhNo215	1	16 0	n d [b]	0	11 0 5 0	11 0, 5 0
DhNo220	1	17 0	0	0	17 0	17 0
DhNo214	1	20 0	0	0	20 0	20 0
		∑ 131 8[c]				
C Groups with HindIII or SalI sites						
DhNo242	2	10 6	0	0	n d	2 6 2 3, 2 0, 1 8, 1 3 0 6
DhNo210	1	13 2	0	0	7 7, 5 5	7 7 3 0 1 5 1 0
DhNo238	1	14 3	n d	0	n d	6 4, 3 5 1 6 1 2 1 1 0 5
DhNo309	1	15 0	0	0	10 4 2 8, 1 2, 0 6	6 3 4 1 1 7 1 2, 1 1, 0 4, 0 2
DhNo205	>8	15 5	0	3	15 5	9 7 5 8
DhNo233	1	16 2	n d	1	5 0 4 0 2 8, 2 4, 1 4, 0 6	2 8, 2 2, 1 6, 1 5, 1 4 1 1, 0 8, 0 6
DhNo207A	1	16 5	0	1	12 5, 2 8, 1 2	n d
DhNo316	1	16 7	n d	1	n d	4 0 3 4, 2 4, 2 0, 1 7, 1 4 1 2, 0 6
DhNo270	1	17 5	0	0	9 8, 7 7	7 7, 3 6, 2 9, 1 7, 1 6
DhNo224	1	18 0	0	>4	9 0 5 0 4 0	5 8 5 0 4 0 2 4 0 8
DhNo209	1	16 4	0	>4	9 0 7 4	7 4 5 8 2 4 0 8
		∑ 169 9[d]				
		∑ 149 6[e]				

[a] Sets of groups with overlapping restriction fragments are printed in small type

[b] Not determined

[c] If the overlap between groups DhNo324 and DhNo367 is taken into account, these nine groups together represent 119 9 kb of DNA

[d] If the overlap between groups DhNo224 and DhNo209 is taken into account these 11 groups together represent 160 9 kb of DNA

[e] If corrected for overlaps between different groups, all 23 groups together represent 328 7 kb of DNA

HindII cleaves less frequently in DNA containing ayl repeats compared with EcoRI (Fig 1), while SalI specifically cleaves YsI repeats at high frequency (Lifschytz 1987, Wlaschek et al 1988, also see Fig 3) Therefore, the screening of SalI libraries would predominantly yield clones with YsI repeats EcoRI has the disadvantage that it has internal restriction sites in ayl repeats and, to a lesser extent, in YsI repeats Therefore, a large number of clones containing solely ayl or YsI repeats will be recovered from EcoRI libraries

More than 800 clones were initially recovered, and 108 clones were randomly chosen for further analysis For each clone it was determined whether an internal BamHI site was present, whether it contained ayl-associated sequences, and, by its SalI fragment pattern and hybridization pattern to PY9 under stringent conditions, whether it contained ayl or YsI repeats These criteria allowed these clones to be assigned to 58 groups

Class II clones containing repeats of the ayl family In 23 groups of clones we did not find SalI repeats, and we therefore assume that these clones contain ayl repeats (Table 3) as all restriction fragments hybridized to PY9 under stringent conditions (see for example Fig 2B) The Class II clones, together with the clones PY1, PY4 and PY9 of Vogt and Hennig (1983), represent about 365 kb of DNA Only three groups of clones have an internal BamHI site (Table 3A) Of these, DhNo278 contains a 14 6 kb sequence of DNA that is homologous to the DNA surrounding the internal BamHI site of DhNocos7 as far as the restriction map can identify sequence homology Of the other two, none was found

Table 4. PY9-hybridizing clone groups from the EcoRI-/EMBL4 library with SalI repeats

Group	Members	Insert (kb)	BamHI sites	HindIII sites	EcoRI fragments (kb)	Fragments from EcoRI-SalI digest (kb)
A groups without HindIII sites						
DhNo200	3	11 4	0	0	5 1, 2 4, 1 55, 1 2, 1 15	0 5, 0 7, 1 0, 1 15, 1 2, 1 6
DhNo291	1	12 2	0	0	4 2, 3 5, 2 5, 1 6	0 5, 1 0, 1 4
DhNo287	2	13 5	0	0	13 5	0 4, 0 6, 0 9, 1 0, 1 1, 1 3
DhNo207B	1	14 5	0	0	14 5	0 4, 0 7, 0 9, 1 2, 2 4
DhNo311[a]	2	14 5	0	0	5 3 2 4 2 0 1 6 1 4 1 2 0 5 5	0 5 5 0 8
DhNo203	1	12 0	0	0	5 3 2 4 1 6 1 2 1 0 0 5 5	0 5 5 0 8
DhNo266	2	15 0	0	0	15 0	0 4, 0 5, 0 8
DhNo277	3	16 0	0	0	16 0	0 6, 1 1, 1 15, 1 5, 1 8, 2 7, 4 2
DhNo229	2	17 0	0	0	14 0–3 0	0 5, 0 9, 1 1, 1 2, 1 4
DhNo225	3	17 0	0	0	17 0	0 4 0 6, 1 1, 1 15, 1 5, 1 8, 3 4
DhNo218	1	17 1	0	0	9 5 3 5 2 3 1 8	0 5 5 0 8 1 0
DhNo363	1	13 0	0	0	9 5 3 5	0 5 5 0 8 1 0
DhNo286	2	18 0	0	0	18 0	0 6, 0 8, 1 0, 1 1, 1 3, 1 7, 2 0
DhNo360	1	18 8	0	0	17 0, 1 2, 0 6	0 25 0 4 0 8
DhNo289	2	18 2	0	0	17 0 1 2	0 25 0 4 0 8
DhNo388	1	17 6	0	0	17 0 0 6	0 25 0 4 0 8
DhNo327	10	17 0	0	0	17 0	0 25 0 4 0 8
DhNo354	1	>16 0	n d [b]	0	3 7, 2 7, 2 6, 2 4, 1 7, 1 4, 1 1, 1 0	0 4, 0 5, 0 8, 1 1, 1 5
DhNo317	1	n d [c]	n d	0	n d	0 55, 0 8, 1 25
DhNo345	1	n d [c]	n d	0	n d	0 55, 1 1
		∑ 309 8[d]				
B Groups with at least one HindIII site						
DhNo395	1	13 4	0	1	12 7 0 7	0 4 0 6 0 8 1 5 2 2 2 8
DhNo321	2	12 7	0	1	12 7	0 4 0 6 0 8 1 5 2 2 2 8
DhNo226	2	14 0	0	1	14 0	0 55 0 95, 1 0, 1 25, 1 35
DhNo397	1	15 2	0	1	7 5 3 4 2 8 1 5	0 4 0 55 1 0
DhNo245	1	14 1	0	1	7 5 2 8 1 8 1 0 0 8	0 3 0 55 1 0
DhNo352	2	16 3	0	>3	7 3, 2 1, 1 8, 1 7, 1 5, 1 3, 0 6	0 25, 0 55 0 95
DhNo230	>4	17 6	0	1	16 0 1 6	0 25 0 4 0 6 1 1 1 4 1 5 1 8
DhNo204	>5	16 0	0	1	16 0	0 25 0 4 0 6 1 1 1 4 1 5 1 8
DhNo284	1	18 6	0	1	11 0 6 0 1 6	0 55 0 9 1 0 1 2 1 25 1 45
DhNo255	1	17 0	0	1	11 0 6 0	0 55 0 9 1 0 1 2 1 25 1 45
DhNo386	1	19 5	0	1	14 0 5 5	0 5 0 9 0 95, 1 0, 1 05, 1 1
DhNo290	1	20 3	0	1	10 0, 5 6, 2 4, 1 5, 0 8	0 5 0 7, 0 8, 3 4, 4 4
DhNo400	1	>11 0	0	2	3 4, 2 5, 1 9, 1 8, 0 7, 0 4	0 55, 0 75, 1 4
DhNo236	1	>17 0	n d	1	3 3, 3 0, 2 5, 2 1, 2 0, 1 9, 1 7, 1 4	0 6, 1 0
DhNo265	1	n d [c]	n d	2	n d	0 4, 0 8, 1 2
		∑ 235 0[e]				
		∑ 544 8[f]				

[a] Sets of groups with overlapping restriction fragments are printed in small type

[b] Not determined

[c] An average insert size of 15 5 kb is assumed for DhNo265, DhNo317 and DhNo345

[d] If corrected for overlaps between different groups, these 20 groups together represent 233 kb of DNA

[e] If corrected for overlaps between different groups, these 15 groups together represent 178 8 kb of DNA

[f] If corrected for overlaps between different groups, all 35 groups with SalI repeats together represent 411 8 kb of DNA

to overlap with any of the clones recovered from the BamHI libraries

Another 9 groups of EcoRI clones have no internal sites for BamHI, HindIII and SalI (Table 3B) These clones account for 120 kb of DNA mainly or exclusively composed of ay1 sequences They probably originate from the megabase cluster of uninterrupted ay1 repeats recently reported by Trapitz et al (1992) This cluster is cleaved in two large fragments by BamHI, in four large fragments by HindIII and by SalI, but in many small fragments by EcoRI (Trapitz et al 1992)

In addition, 11 groups of clones without BamHI sites were recovered. They have internal SalI or HindIII sites (Table 3C) Together they represent 170 kb of DNA It

is possible that these groups of clones contain ay1-associated DNA sequences, but this was not further investigated by more detailed restriction mapping and hybridization experiments

Class III clones containing repeats of the Ysl family In 35 groups, a regular, group-specific SalI fragment pattern was obvious (Table 4, Fig 5), indicating a tandem arrangement of SalI repeats Since all SalI fragments of these groups hybridized only very weakly to PY9 under stringent conditions (see for instance Fig 2C), they contain repeats of the Ysl family (Wlaschek et al 1988) Together, all clones contain about 410 kb of DNA from the short arm of the *Y* chromosome Because none of these clones contained restriction fragments that failed to hybridize to PY9, repeats of the Ysl family do not seem to be interspersed by other, unrelated sequences, in contrast to repeats of the ay1 family Sequence analysis of 0 55 kb SalI fragments from group DhNo255 and of 0 25 and 0 4 kb fragments from group DhNo327 confirmed that these fragments belong to the Ysl family (R Hochstenbach, M Knops and W Hennig, data not shown)

The 20 groups of clones that lack a HindIII site (Table 4A) might originate from the extended cluster of Ysl repeats that is cleaved in two large fragments by BamHI and in four large fragments by HindIII (Trapitz et al 1992) In addition, 15 groups of clones, together representing 180 kb of DNA, have at least one internal HindIII site (Table 4B)

In conclusion, from an EcoRI library only clones consisting either of homogeneous ay1 or homogeneous Ysl sequences were obtained Although we cannot exclude that some of the Class II clones contain other sequences in addition to ay1, we consider this an unlikely possibility, since, using the same mapping approach, such ay1-associated sequences were readily detected in the Class I clones Only 1 out of 108 EcoRI groups was found to overlap with a group from the BamHI collection Thus, screening EcoRI libraries with PY9 is not an efficient way to isolate clones that extend or connect the Class I clones Apparently, BamHI sites are much rarer in EcoRI fragments that contain ay1 than would be expected from a random distribution of BamHI sites in these fragments

Separate organization of ay1 and Ysl on the short arm of the Y chromosome

The analysis of the different clone classes indicates that ay1 and Ysl repeats are not intermingled, since we did not find any clone containing repeats of both families in more than 1000 kb of genomic DNA analysed In addition, we did not find a single Class III clone containing Ysl repeats, of which the restriction pattern overlapped with that of any of the Class I or Class II clones containing ay1 repeats We therefore studied the distribution of ay1 and Ysl repeats on Southern blots of genomic DNA As a specific probe for the ay1 family we used the original ay1 repeat as defined by Vogt and Hennig (1986a) As a specific probe for the Ysl family we used a 562 bp SalI DNA fragment from Class III clone DhNo255 This fragment has 91% homology to the Ysl10 20 clone sequenced by Wlaschek et al (1988), but only 71% to the original ay1 sequence of Vogt and Hennig (1986a) (R Hochstenbach, M Knops and W Hennig, data not shown) For cleaving genomic DNA we used the enzymes SalI and XbaI, which both have conserved cleavage sites in Ysl repeats but not in ay1 repeats (Wlaschek et al 1988)

Under non-stringent conditions both the ay1 and the Ysl-specific probe hybridize to DNA fragments of similar sizes in SalI or XbaI digested genomic DNA (Fig 3, left panel), indicating cross-hybridization between repeats of both families Similar patterns were obtained by Vogt et al (1982) using PY9 However, under stringent conditions (Fig 3, right panel) it appears that the majority of ay1 repeats are in DNA segments that are not resolved in the agarose gel, indicating that these fragments are almost completely refractory to cleavage by SalI and XbaI Some ay1 repeats however, must be in DNA segments that are cleaved by SalI, as shown by the restriction mapping of the Class I clones (Fig 4) and of several Class II clones (Table 3), but as these repeats are almost undetectable on Southern blots, they represent a minority of all ay1 repeats In contrast, Ysl repeats are absent in the large uncleaved DNA fragments containing ay1, since most if not all, Ysl repeats are found in segments of DNA that are frequently cleaved by both enzymes The major SalI and XbaI fragment length is 0 55–0 6 kb, which is also seen in many of the

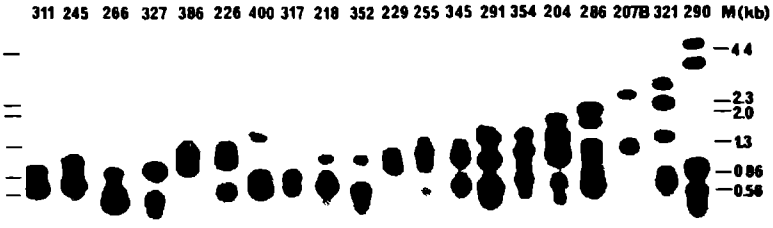


Fig. 5. Southern blot of 20 different Class III Ysl clone groups from the EcoRI lambda library DNA of these clones was digested with EcoRI and SalI separated on agarose gels transferred to nylon membranes and hybridized with ^{32}P nick translation labelled PY9 insert under non stringent conditions, similar to those used for the genomic blot of Fig 1 PY9 hybridized to all SalI fragments seen on the gel

Class III clones (Table 4, Fig 5) The residual YsI hybridization to large DNA fragments most likely results from partial cleavage of the genomic DNA, since under identical conditions there is no hybridization of the ay1-specific probe to the 0 55 0 6 kb YsI repeats

Thus consistent with the analysis of the genomic clones, the hybridization patterns of ay1 and YsI-specific probes on Southern blots of genomic DNA imply that repeats of the ay1 and YsI families are not intermingled and are located in non-overlapping domains of the *Y* chromosome

Evidence for the genomic authenticity of the clones

A serious concern in analysing repetitive DNA sequences are artefacts resulting from recombination during the propagation of the cloned DNA sequences in the host cells If large proportions of the clones are artefacts the mapping of the loop would be an impossible task However we have no evidence for instabilities of cloned DNA fragments, as was evident, for example, for clones containing simple satellite repeat sequences of *Drosophila melanogaster* (Brutlag et al 1977) Also Carlson and Brutlag (1977) did not find decreasing insert sizes for cloned tandem repeats of the more complex 359 bp satellite DNA sequence of *D melanogaster*, which in sequence complexity is comparable to that of repeats of the ay1 family

Cases that raise the suspicion of deletions during bacterial propagation are the Class I lambda clone groups DhNo90, DhNo87 and DhNo52 They all have very similar restriction maps and contain the same 5 8 kb BamHI–EcoRI fragment which is flanked by a cluster of ay1 repeats of variable length (Fig 4) The smaller clones could, therefore be derived from the larger ones by successive deletions of ay1 repeats during propagation on host cells like *E coli* NM538 and Q358 that are not recombination deficient (Wyman and Wertman 1987) However, restriction digests never displayed weak bands that could be taken as an indication of clone rearrangements during bacterial propagation This also holds true for the lambda clones containing only YsI repeats Also Wlaschek et al (1988), using the same host-vector system did not report instability of lambda clones with ay1 and YsI repeats

Several other lines of evidence indicate the genomic authenticity of the Class I clone groups DhNo90, DhNo87 and DhNo52, and of other related groups as well The 17 2 kb BamHI fragment of DhNo87 is also present in DhNocos47 (see Table 2) It is therefore very unlikely that clones of the group DhNo87 represent a deletion product of the longer clones in group DhNo52 Further, both DhNo90 and DhNo87 were recovered as independent isolates with opposite orientations in the λEMBL3 vector Also the two members of the DhNo86 group had opposite orientations In addition, in the BamHI Southern blot of genomic DNA shown in Fig 1 BamHI fragments of 4 4, 7 0, 9 0 and 10–11 kb hybridize to PY9 These could very well correspond to the smaller ay1 containing BamHI fragments of the lambda and cosmid clones (Table 2)

As already mentioned, the Class II group DhNo278 from the EcoRI library contains a part of DhNocos7 Also the eight cases of partially overlapping Class III groups of clones from the EcoRI library illustrate the reliability of our cloning procedures For example, group DhNo360 contains EcoRI fragments of 17 0, 1 2 and 0 6 kb Group DhNo289 (see Fig 2C) lacks the 0 6 kb fragment, and group DhNo388 the 1 2 kb fragment, while group DhNo327 lacks both the 0 6 and 1 2 kb fragments (Table 4A)

In conclusion, multiple independent isolates of the same DNA fragment, overlaps between different groups of clones from the same library, and recovery of the same genomic DNA fragment using different host-vector systems, all indicate the authenticity of the cloned DNA fragments Therefore, clone instabilities are not an experimental problem in our case

Different groups of Class I clones contain identical or related ay1-associated sequences

Restriction fragments containing ay1-associated DNA from the outer ends of the BamHI clones are important probes for the isolation of overlapping clones from other libraries However, if such fragments are not unique for one clone clones with cross-hybridizing fragments are not necessarily overlapping We therefore investigated whether the same or similar ay1-associated DNA fragments occur repeatedly within our Class I clone collection by hybridization of selected fragments from one particular clone to restriction fragments of all other clone groups

Group DhNo90 contains an ay1-associated 5 8 kb BamHI–EcoRI fragment (DhNo90BE5 8) that is also present in groups DhNo87 and DhNo52 (Fig 4) The comparison of the restriction maps revealed that this fragment is very similar, but not identical, to an ay1-associated sequence from DhNocos18 Indeed, DhNo90BE5 8 hybridizes to its counterparts in DhNo87, DhNo52 and DhNocos18 under stringent conditions (0 1 × SSC, 65° C) In addition, it hybridized to DhNo19EE3 7, but only under non-stringent conditions (2 × SSC, 65° C) In turn, DhNo19EE3 7 cross-hybridized to DhNocos6HH2 1, and also to DhNo86BH3 8, but again only under non-stringent conditions Thus, out of 17 BamHI clones tested, 7 clones contain a related ay1-associated sequence

Vogt and Hennig (1986b) demonstrated that the PY3 subclone MY3 contained a *Y*-associated sequence that is transcribed in the loop and of which at least seven copies occur on the *Y* chromosome As indicated by comparison of restriction maps (Fig 4) a complete copy of MY3 including both the ay1 repeats and the transcribed *Y*-associated sequence, is included in group DhNocos7 Both copies are however not identical, as revealed by the alignment of corresponding nucleotide sequences (data not shown) In addition group DhNocos6 also contains part of an ay1-associated sequence of MY3, although the restriction maps do not suggest any similarity The comparison of the nucleotide se-

quences however, discloses 92% identity between the shared sequences of MY3 and DhNocos6 (data not shown).

These results prove that ay1-associated sequences occur repeatedly, even within the limited number of cases inspected for homologies. This indicates that ay1-associated sequences in general may be present in multiple copies within the loop forming region, and therefore, the variability of the ay1-associated sequences may be rather limited.

Y-associated sequences from Class I clones are enriched in the chromocentre

As emphasized in the previous sections, identification of overlaps between ay1-containing BamHI clones by screening genomic libraries with outer segments of these clones as probes, may lead to false positives because other cross-hybridizing sequences may be present elsewhere in the genome, therefore representing *Y*-associated sequences. Therefore, each clone recovered in such screens must be tested for its *Y*-chromosomal origin. To determine the extent of this problem, we investigated the genomic distribution of the cross-hybridizing ay1-associated fragments of Class I clone groups DhNo19, DhNo90 and DhNo86 (Figs. 6, 7).

The presence of ay1-associated sequences on other chromosomes can be verified by comparison of their hybridization patterns on Southern blots of DNA from males and females, or directly by in situ hybridization on polytene chromosomes of salivary gland cells. If such sequences are located in underreplicated heterochromatin, metaphase chromosomes must be used for the in situ hybridization.

In polytene chromosomes, DhNo19EE3.7 hybridizes to two bands in the euchromatin (Fig. 6A). According to the map of Berendes (1963), these bands are 58D–59A on chromosome *3*, and 80B–C on chromosome *4*. The hybridization to Southern blots of HindIII-digested genomic DNA of females indicates that at least two copies of this sequence are not located on the *Y* chromosome. However, several male-specific fragments are seen as well (Fig. 7A). Therefore, this sequence belongs to the type designated as *Y* associated.

On Southern blots of DNA from females of other wild-type strains of *D. hydei* (Zürich, Alicante and Madeira) two to four HindIII fragments larger than 7 kb are seen (data not shown). This demonstrates that the number of copies of this sequence that are not on the *Y* chromosome varies between different wild-type strains. Since all euchromatic copies in all strains share a 3.7 kb EcoRI fragment, at least a part of this sequence is conserved. The EcoRI fragment in DhNo19 must, however, be of *Y*-chromosomal origin as it is associated with the *Y*-specific ay1 repeats.

Also DhNo90BE5.8 and DhNo86BH3.8 are *Y*-associated sequences but they have a distribution in the genome quite different from that of DhNo19EE3.7. In addition, they were found at higher copy numbers. DhNo90BE5.8 is present only in the centromere-associated heterochromatin of the *X* chromosome and also

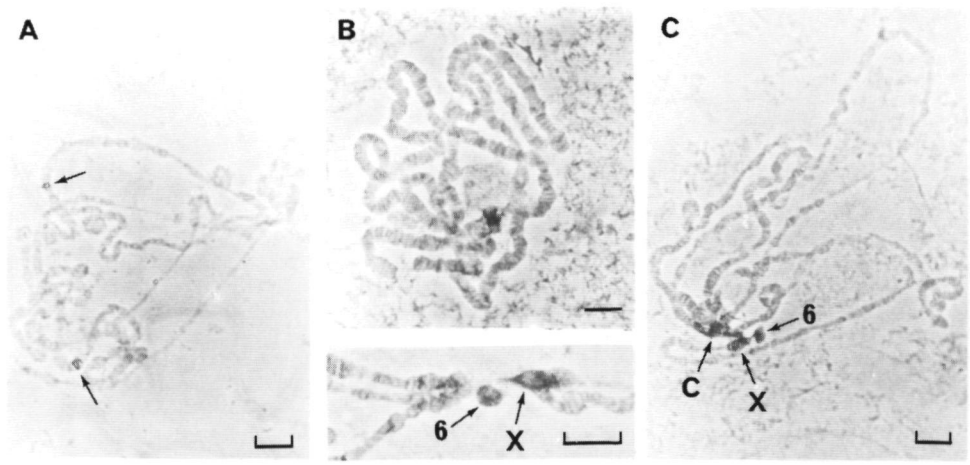
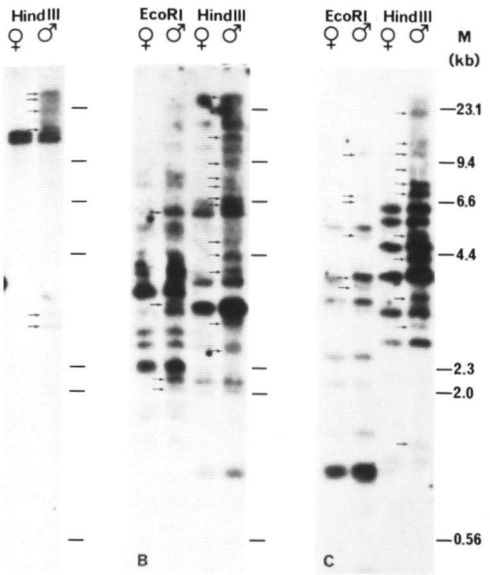


Fig. 6A–C. In situ hybridization of three related *Y*-associated sequences to polytene chromosomes from salivary gland cells from female third instar larvae of the Tübingen wild-type strain of *Drosophila hydei*. Single-stranded RNA probes were labelled with digoxigenin UTP (DIG-UTP) and visualized using an anti-DIG antibody conjugated with alkaline phosphatase. The hybridization patterns of the following fragments are shown (phase contrast): **A** DhNo19EE3.7. There are two copies in the euchromatin (see text), which are indicated by *arrows*. **B** DhNo90BE5.8. Signals are seen throughout the small autosome *6* and throughout the pericentric heterochromatin of the *X* chromosome, and are indicated by *arrows marked 6* and *X*, respectively. There are no signals on the large autosomes. **C** DhNo86BH3.8. Signals are seen throughout chromosome *6* (indicated by an *arrow marked 6*), in the centromere-associated heterochromatin of the *X* chromosome and in that of the four large autosomes. The *X*-chromosomal heterochromatin (indicated by an *arrow marked X*) can be seen separated from that of the other large chromosomes in the chromocentre (indicated by an *arrow marked C*). Bar represents 10 μm



A–C. Southern blots of genomic DNA hybridized with the Y-associated sequences as in Fig. 6. Genomic DNA from and male adults was cleaved by EcoRI or HindIII, separated % agarose gel, immobilized on nylon membranes, and hyd with the following ^{32}P nick translation-labelled restriction nts: **A** DhNo19EE3.7, **B** DhNo90BE5.8, **C** DhNo86BH3.8. ranes were washed in 2×SSC at 65° C, allowing cross-hytion between these related sequences (see text). However, ee hybridization patterns are all completely different since robe hybridized much more strongly to homologous se- s in the genome than to less related sequences from the clone groups. Some *Y*-chromosomal restriction fragments icated by *arrows*. In addition, several fragments shared beDNA from both sexes show much stronger hybridizaton anes containing DNA from males, and since equal amounts A was loaded in all lanes, these also indicate *Y*-chromosomal nts

ghout chromosome *6* (Fig. 6B). DhNo86BH3.8 is it in the centromere-associated heterochromatin of e large chromosomes and throughout the small iosome *6* (Fig. 6C). Both DhNo90BEH5.8 and 86BH3.8 display no additional hybridization sig- 1 the euchromatic parts of the large chromosomes. ime results were obtained with the Zürich, Alicante Madeira wild-type strains (data not shown). Com- n of the hybridization patterns on Southern blots NA from males and females shows that both of *Y*-associated sequences have at least ten copies *Y* chromosome (Fig. 7B, C).

conclusion, the hybridization of each of these ay1- ated fragments to male-specific restriction frag- adds further evidence to the *Y*-chromosomal ori- the BamHI clones containing interspersed ay1 s. Because each ay1-associated fragment is present ier chromosomes as well, these ay1-associated se- es are not *Y* specific but *Y* associated. The genomic ution of this limited sample of *Y*-associated sequences suggests that it may be a common phenor that ay1-associated fragments are also located on chromosomes. Therefore, cross-hybridization be sequences of *Y*-chromosomal origin with relat quences on other chromosomes will seriously i the search for overlaps between the BamHI clon contain such *Y*-associated sequences. This require laborious procedures in the construction of contig

Y-associated sequences from Class I clones are specifically transcribed in the Nooses

If Class I clones containing ay1 repeats and *Y*-asso sequences represent segments of the *Nooses*, *Y*-asso sequences from these clones must be present in loo scripts. We therefore performed transcript in situ h ization experiments on fixed primary spermatocyt ing wild-type males and males lacking the short arm *Y* chromosome, which therefore lack the *Nooses* brush loop pair. The lampbrush loops of a wil male are shown in Fig. 8A. As probes we used the hybridizing *Y*-associated fragments DhNo90BE5. DhNo86BH3.8 which were subcloned in pBluesc KS vectors. For comparsion of the hybridizatio terns obtained with these probes to that of ay1, w subcloned DhNo19BE2.2, a fragment containing a peats from one of the Class I clones. Strand-speci goxygenin-labelled RNA probes were prepared fro earized plasmids by in vitro transcription using T3 or T7 polymerase.

As expected, the ay1-containing DhNo19BE2.2 ment hybridizes strand-specifically with transcripts *Nooses* lampbrush loop pair (Fig. 8B, C), consisten the results of Lifschytz and Hareven (1985) and T et al. (1988). Thus, all ay1 repeats that are trans in the loop have the same polarity. Similar result obtained with the *Y*-associated fragments DhNo90 (Fig. 8D, E) and DhNo86BH3.8 (Fig. 8G, H). In of these cases also, only one strand hybridized to scripts of the *Nooses* loop pair, indicating that all of these sequences that are transcribed in the loop the same orientation. The labelling patterns of bo associated sequences cover the entire transcriptior and are highly similar to those obtained using p containing ay1 repeats. This indicates, firstly, that are multiple copies of these sequences within the and secondly that, throughout the entire loop, thes ies are intermingled with co-transcribed ay1 repeats sistent with the organization of these sequences in s Class I clones (Fig. 4). Since under identical hybr tion conditions both DhNo90BE5.8 and DhNo86I have different localization patterns on polytene ch somes, it is highly unlikely that the hybridizati *Nooses* transcripts is the result of cross-hybridizat one fragment to transcripts of the other.

In order to demonstrate that the hybridizati both *Y*-associated sequences to loop transcripts i mary spermatocyte nuclei of wild-type males is s for the *Nooses*, we repeated the transcript in situ h

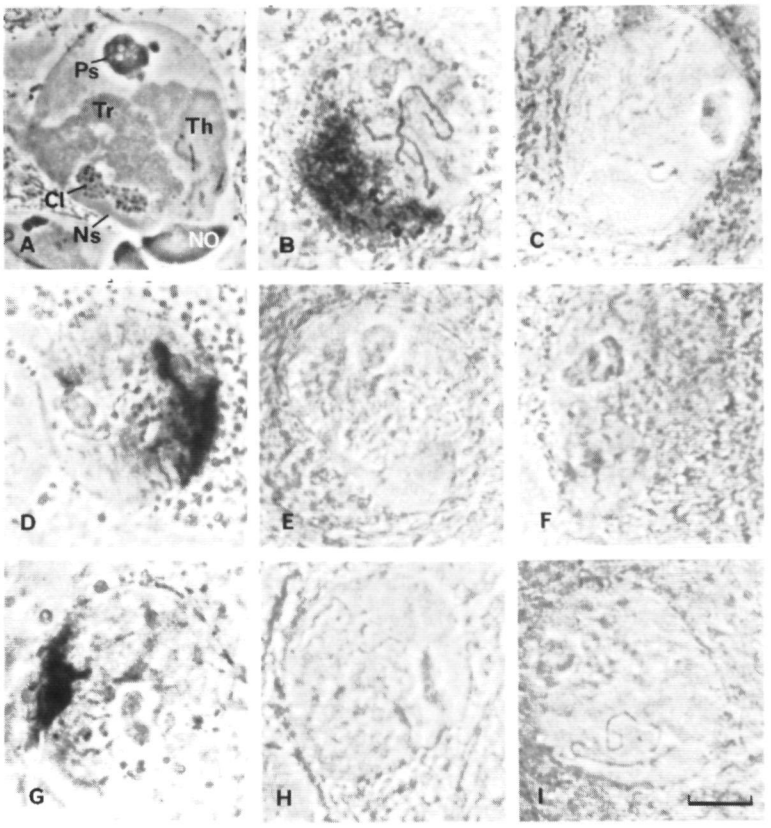


Fig. 8A–I. Transcript in situ hybridization of subcloned fragments from Class I clones to fixed primary spermatocyte nuclei of *Drosophila hydei*, using single-stranded RNA probes labelled with DIG-UTP. Phase contrast. **A** Living primary spermatocyte nucleus of a wild-type male. All five lampbrush loop pairs are indicated: *Th Threads*, *Ps Pseudonucleolus*, *Tr Tubular ribbons*, *Cl Clubs* and *Ns Nooses*. The nucleolus organizer (*NO*) is indicated as well. **B** and **C** Wild-type primary spermatocyte nuclei showing hybridization of strand-specific probes for the ay1-containing DhNo19BE2.2 fragment. Only one strand of ay1 is present in transcripts of the *Nooses*. **D** and **E** Wild-type primary spermatocyte nuclei showing hybridization of strand-specific probes for the *Y*-ass DhNo90BE5.8 sequence. Only one strand of this sequence ent in *Nooses* transcripts. In **F**, the same probe as in **D** was ized to primary spermatocyte nuclei of GE7 males. In the of the *Nooses*, there is no hybridization to other loop p and **H** Wild-type primary spermatocyte nuclei showing hyb tion of strand-specific probes for the *Y*-associated DhNo8 sequence. Again, only one strand of this sequence is tran in the *Nooses*. In **I**, the same probe as in **G** was hybrid primary spermatocytes of GE7 males, and again there is n on any other loop pair. Bar represents 10 μm

ization, but now using primary spermatocytes of (sterile) GE7 males that lack the short arm of the *Y* chromosome. Such males therefore have no *Nooses* loop pair in their primary spermatocyte nuclei. The hybridizing strands of both DhNo90BE5.8 (Fig. 8F) and of DhNo86BH3.8 (Fig. 8I) do not hybridize to transcripts of any of the four remaining loop pairs in GE7 males. This indicates that these sequences are specifically transcribed in the *Nooses*, and not in any other loop pair.

In conclusion, the *Y*-associated fragments of the Class I clones DhNo90 and DhNo86 are specifically transcribed in the *Nooses* lampbrush loop pair. This result strongly suggests that these clones represent segments of the DNA that is transcribed in this loop pair.

Discussion

ay1 repeats are not restricted to the region forming the Nooses loop on the D. hydei Y chromosome

Our approach to reconstitute the lampbrush loo *Nooses* has indicated that the region of the *Y* ch some accommodating the lampbrush loop, if defi DNA sequence composition, is much more ext than the 260 kb length estimate of the transcriptio This estimate is based on two independent me ments: first on the cytological length of the *Nooses* in fully developed primary spermatocytes (see H et al. 1974 and also Fig. 5C of Hennig 1987), and s

on Miller spreading of transcribed chromatin (Grond et al 1983) By cytology, the length is estimated to be 50 µm or less, in the Miller spreading it was determined as 50 µm It could be argued that the loop visualized by electron microscopic (EM) techniques is in an incompletely extended state as is expected for young primary spermatocytes However, the coincidence of the size values obtained by entirely different means makes this unlikely Therefore, an estimate of 260 kb for the transcription unit must be close to the real size It most likely represents a maximum value since it was calculated using the conventional assumption that 1 µm of transcribed DNA in the spread loop chromatin corresponds to 5 2 kb of DNA (see Laird et al 1976) Moreover, based on the relation between the density of RNA polymerases and the amount of DNA per micron, it seems even more appropriate that a value of 4 7 kb/µm should be used, predicting a loop length of about 240 kb (see discussion by Hager and Miller (1991))

Table 5 summarizes our current knowledge of the different types of repetitive DNA sequences on the short arm Using as a probe ay1 repeats, which are specifically transcribed in the *Nooses*, we have recovered 158 different genomic clones, which together contain more than 1 000 kb of DNA from the region where the loop is located The size of this region may even exceed 2000 kb (Table 5)

All evidence indicates a rather restricted DNA sequence heterogeneity within the genomic clones and, assuming that these clones are a representative sample, also within the loop-forming region on the *Y* chromosome Three main types of repetitive DNA sequences were identified in our clones the ay1 family of repeats, the YsI family of repeats, which is derived from ay1 sequences, and a more heterogeneous repetitive DNA that has been designated as *Y*-associated because some copies of it are located on chromosomes other than the *Y* chromosome This *Y*-associated DNA is exclusively associated with ay1, since it was not detected in more than 400 kb of DNA containing YsI repeats Our present study also revealed that within the *Y*-associated DNA sequences the sequence heterogeneity is less than expected, since such DNA sequences from different clones were shown to cross-hybridize They may belong to one family of repetitive DNA sequences

In calculating the total length of these three types of DNA sequences we have no means of assessing whether any fragment occurs in multiple identical copies within the short arm of the *Y* chromosome From all Southern blotting and sequencing data available to us we assume, however, that a frequent repetition of identical DNA sections is highly unlikely

Thus, it is evident that the total amount of DNA on the short arm of the *Y* chromosome containing ay1

Table 5 Repetitive DNA sequences on the short arm of the *Drosophila hydei Y* chromosome[a]

Family of repetitive DNA sequences	Sequence complexity	Approximate amount of DNA	Reference
Interspersed ay1 repeats	< 300 kb	> 300 kb	This paper
Homogeneous ay1 repeats	400 bp	500 kb 815 920 kb[b] > 325 kb[c]	Wlaschek et al (1988) Trapitz et al (1992) This paper
Homogeneous YsI repeats	600 bp	1 500 kb 630–710 kb[b] > 410 kb	Wlaschek et al (1988) Trapitz et al (1992) This paper
PY5 TaqI repeats[d]	180 bp	n d [e]	Vogt and Hennig (1983) Vogt et al (1986)
PY8[f]	3 kb	n d [e]	Vogt and Hennig (1983) Vogt et al (1986)
Ribosomal RNA genes[g] without IVS with IVS	 11 kb[h] 17 kb[h]	 550 kb n d	Hennig et al (1975) Meyer and Hennig (1974) Hennig Vogt, ten Hacken (unpublished data)[i]

[a] The entire *Y* chromosome is about 43000 kb (Zacharias et al 1982) and as estimated from well spread metaphases the short arm contains approximately 6000 kb of DNA

[b] These numbers are not derived from the analysis of DNA from whole animals but from the KUN-DH-33 cell line which contains a *Y* chromosome

[c] Including PY1 PY4 and PY9 of Vogt and Hennig (1983)

[d] Not much is known about the PY5 sequences except that they are cleaved by TaqI and not by most other restriction enzymes The regular spacing of TaqI sites at approximately 180 bp suggests a satellite-like character of this sequence There is no cross hybridization to ay1

[e] The strength of the signal on metaphase chromosomes suggests that this sequence is less abundant than the combined lengths of ay1 and YsI

[f] PY8 is cleaved by SalI and BglII but not by at least 13 other restriction enzymes There is no cross hybridization to ay1

[g] Different wild type strains may have different numbers of rRNA genes in the nucleolus organizer of the short arm, or may lack the IVS in the rRNA cistrons of the *Y* chromosome (Kunz et al 1981)

[h] The length of an rRNA cistron without IVS is 11 kb if the 6 kb-long IVS is present in the 28S coding region the length is 17 kb (Renkawitz et al 1980 Hennig et al 1982)

[i] Probes containing only IVS sequences hybridize in situ to the terminal end of the short arm of the *Y* chromosome in metaphase spreads prepared from males of the Tubingen wild type strain

or YsI repeats considerably exceeds the size of the *Nooses* lampbrush loop. The question therefore arises how the sequences that are transcribed in the loop can be identified. Before addressing this question we will first discuss the arrangement of the different sequences at the DNA level.

The organization of ay1 and YsI repeats on the short arm of the Y chromosome

From the analysis of clones from the EcoR1 library, it emerges that ay1 sequences and YsI sequences occur in large runs of tandem repeats that are uninterrupted by other sequences. This is in agreement with the studies of Wlaschek et al. (1988) and Trapitz et al. (1992). Our data do not allow extensive conclusions on the physical sizes of these large ay1 and YsI sequence blocks, but from pulsed-field blots of DNA from a cell line containing the *Y* chromosome, Trapitz et al. (1992) estimate a size of 815 920 kb for the ay1 cluster and 630–710 kb for the YsI cluster. Several of the Class II clones described here may originate from the large ay1 cluster, and the Class III clones may account for more than 400 kb of the YsI cluster.

Probes that are specific for ay1 and YsI both hybridize to a central position on the short arm of the *Y* chromosome (Vogt and Hennig 1983; Lifschytz et al. 1983; Wlaschek et al. 1988). However, using Hoechst 33258-banded metaphase chromosomes, K.H. Glätzer (cited by Trapitz et al. 1992) has shown that the YsI cluster is in a more proximal position than the ay1 cluster. In the accompanying paper, we confirm this result using two-colour fluorescence in situ hybridization, and in addition, we demonstrate that both clusters are clearly separated and non-overlapping in interphase chromatin (Hochstenbach et al. 1993).

The organization of ay1 and YsI repeats in separate clusters explains the earlier results of Vogt et al. (1982), who showed that hybridization of PY9 under conditions that allow cross-hybridization to YsI detects two distinct fractions of genomic DNA in SalI and XbaI digests: one fraction that remains uncleaved, and another that is cleaved into many small fragments. Here, we show that both fractions can be discriminated by hybridization under stringent conditions: the uncleaved fraction contains the ay1 repeats, the small SalI and XbaI fragments contain the YsI repeats.

ay1-associated and Y-associated DNA sequences

DNA fragments from the Class I clones, combining ay1 repeats as well as DNA sequences of a different character, deserve particular interest since ay1-associated sequences have been demonstrated to be transcribed in the *Nooses* loop pair, as for instance the *Y*-associated part of MY3 (Vogt and Hennig 1986b). The 17 different BamHI Class I clones represent a total DNA heterogeneity of approximately 300 kb if the restriction maps are used as a criterion of sequence heterogeneity. The actual sequence complexity is, however, considerably less if the repetitivity of the ay1 sequences as well as that of the ay1-associated sequences are taken as a criterion, and the divergence of the repeats is neglected. As can be estimated from the restriction maps, at least one-third of the DNA in these clones is ay1-associated (Table 2). From our data we cannot yet decide how many different repeated DNA sequence families contribute to the ay1-associated DNA sequences, but the cross-hybridization between *Y*-associated DNA sequences of many of the Class I clones (Fig. 4) suggests a restricted heterogeneity at this level as well.

We also do not know how much DNA containing interspersed ay1 repeats is present on the short arm of the *Y* chromosome, nor do we know which fraction of the interspersed repeats is represented by the 300 kb of Class I clones. Assuming that the ay1-containing BamHI fragments that can be separated on normal agarose gels (Fig. 1) also contain other DNA sequences, the amount of interspersed ay1 repeats is considerably less than the combined amounts of homogeneous ay1 and YsI repeats that are not cleaved by BamHI.

The failure of other investigators to recover such interspersed DNA fragments can easily be explained by their choices of restriction enzymes for library construction. Lifschytz et al. (1983) have used an EcoRI library and Wlaschek et al. (1988) used a Sau3A library. Both enzymes cleave ay1 and YsI repeats at high frequency, and there are conserved sites for EcoRI in ay1, and for Sau3A in both ay1 and YsI (Vogt and Hennig 1986a; Wlaschek et al. 1988). This also explains why in our screen of the EcoRI library clones with homogeneous ay1 (Table 3) or YsI repeats (Table 4) were preferentially recovered, and outnumber the DNA fragments composed of ay1 repeats and ay1-associated DNA.

In our present study we have shown that some of these ay1-associated DNA sequences are *Y*-associated because they are also found on other chromosomes (Figs. 6, 7). It could be argued therefore that clones containing such *Y*-associated sequences are not necessarily of *Y*-chromosomal origin. This is however, highly unlikely, since ay1 does not hybridize at all on Southern blots of genomic DNA from females, indicating that ay1 repeats occur exclusively on the *Y* chromosome (Vogt et al. 1982; Vogt and Hennig 1986a; Wlaschek et al. 1988). Further evidence for the *Y*-chromosomal origin of these clones is provided by their hybridization to male-specific restriction fragments seen on Southern blots of genomic DNA (Fig. 7), and in addition, by their hybridization to transcripts of the *Nooses* (Fig. 8).

Identification of the sequences that are transcribed in the Nooses: co-transcription of ay1 repeats and Y-associated sequences

For the reconstruction of the DNA of the *Nooses* transcription unit it is an essential prerequisite that transcribed sequences can be discriminated from those that are not transcribed. Although it is evident that ay1 and YsI probes of a few kilobases length hybridize to loop

transcripts, this does not prove that repeats of both families are expressed as transcripts nor that the entire loop is composed of such repeats as was recently proposed by Trapitz et al (1992) In fact, the 1450 1630 kb estimate for the combined length of the ay1 and YsI clusters claimed by these authors excludes the possibility that they are transcribed in their entirety in the loop By application of stringent hybridization conditions a distinction between transcribed and non-transcribed families of repetitive DNA sequences can be made, showing that repeats of the YsI family are not transcribed (R Hochstenbach, M Knops and W Hennig, unpublished data)

The observation that the *Y*-associated sequence of MY3 hybridizes to *Nooses* transcripts (Vogt and Hennig 1986b), was the basis of the interspersion model for the organization of the DNA sequences in the loop (Hennig 1987, 1990 Hennig et al 1989) At the sequence level the model was supported only by a single clone MY3 that could account for not more than 1% of the loop DNA However the interspersion model now rests on much firmer ground Firstly, in 17 different Class I clone groups, we have recovered sufficient DNA that is organized similarly to MY3, to accommodate the entire 260 kb of the *Nooses* transcription unit Secondly we have shown that in addition to the *Y*-associated sequence of MY3, the *Y*-associated sequences DhNo86BH3 8 and DhNo90BE5 8 are also specifically transcribed in the *Nooses* loop pair (Fig 8) The labelling patterns of these fragments after transcript in situ hybridization suggest that they are present in multiple copies that are dispersed throughout the entire loop Consistent with this, Southern blots of genomic DNA indicate that there are multiple copies of both sequences on the *Y* chromosome (Fig 7B and C), and in addition, we have recovered four different Class I clones containing the DhNo90BF5 8 fragment Thirdly the simultaneous hybridization of ay1 and DhNo90BE5 8 to *Nooses* transcripts results in identical hybridization patterns indicating the intermingling of both sequences throughout the transcription unit (R Hochstenbach, R Suikerbuijk and W Hennig, unpublished data)

We do not known which fraction of the loop DNA is represented in our collection of Class I clones However by combining the lengths of the Class I clones that contain transcribed *Y*-associated sequences, it seems that about 100 kb of the loop DNA may be represented by PY3, DhNo86 DhNo90 DhNo52, DhNo87 and DhNocos18 As indicated by their restriction maps, these clones contain almost as much ay1-associated sequences as ay1 repeats, and therefore a substantial fraction of the transcription unit may consist of sequences unrelated to ay1 Remarkably, all copies of the *Y*-associated DhNo86BH3 8 and DhN90BF5 8 sequences within the loop have the same polarity which was also demonstrated for the transcribed *Y*-specific repeats within this and in other loop pairs of *D hydei* (Lifschytz and Hareven 1985 Trapitz et al 1988) The functional significance of this phenomenon is unclear

Since the amount of DNA on the short arm of the *Y* chromosome containing interspersed ay1 repeat blocks is larger than the loop we cannot draw final conclusions as to which of the recovered Class I clones are parts of the *Nooses* transcription unit It is however possible to establish rules for a distinction between transcribed and non-transcribed DNA segments from our collection of Class I clones These rules will permit the assignment of distinct cloned DNA fragments to the transcription unit (R Hochstenbach, H Harhangi, K Schouren and W Hennig in preparation) A final identification of all sequences that are transcribed in the *Nooses* may however only be possible if a physical map of the entire region of the *Y* chromosome surrounding the transcription unit is available

We have started to investigate the nature of the transcribed *Y*-associated DNA sequences Interestingly, the *Y*-associated sequence of MY3 (Vogt and Hennig 1986b), as well as the DhNo90BE5 8 sequence (Hochstenbach et al 1983b) were found to have significant sequence similarity to the *gypsy* retrotransposon known from *D melanogaster* and *Drosophila virilis*, and we have detected similar *gypsy*-like sequences in other transcribed *Y*-associated fragments of the Class I clones as well (R Hochstenbach H Harhangi K Schouren and W Hennig in preparation) We therefore speculate that the *Y*-associated DNA sequences in the lampbrush loop pair *Nooses* are to a large extent represented by a few families of transposable elements or even belong to only one family In this context it is noteworthy that we have earlier identified transcribed *Y*-associated sequences from the loops *Threads* and *Pseudonucleolus* as retrotransposons of the *micropia* family (Huijser et al 1988)

The organization of YsI and ay1 sequences on the Y chromosome of a cell line may not be representative of that on the wild-type Y chromosome in flies

Although the general organization of the ay1 and YsI sequences is now fairly well established the organization of ay1 and YsI repeats on the *Y* chromosome of the KUN DH-33 cell line as recently described by Trapitz et al (1992), displays a number of discrepancies with data reported in this paper Firstly it is puzzling that in BamHI digests of the cell line DNA, ay1-containing fragments in the 10 to 40 kb range as seen in genomic DNA from flies (Fig 1) and in our clones (Fig 4) could not be detected in DNA from the KUN-DH-33 cell line even after prolonged exposures This suggests that during establishment or maintenance of the cell line the *Y* chromosome may have preferentially lost the interspersed ay1 repeats or the entire region with such DNA sequences

A second discrepancy concerns the organization of the uninterrupted YsI repeats In HindIII digests of cell line DNA that were separated by pulsed-field gel electrophoresis, only four large fragments of 310 210 110 and 80 kb were found to hybridize to a YsI probe Smaller HindIII fragments containing YsI were not detected (Trapitz et al 1992) The cumulative length of the large fragments was considered to set an upper limit of 710 kb to the amount of uninterrupted YsI repeats However,

from the EcoRI lambda library we recovered 15 clone groups from the YsI family with at least one HindIII site (Table 4B). Since these 15 groups together contain about 180 kb of DNA, which must be added to the combined length of the large fragments, there must be considerably more YsI repeats on the *Y* chromosome of flies than indicated by the analysis of DNA from the cell line. In fact, this places the estimate for the actual amount of YsI closer to the 1 500 kb claimed earlier by the same investigators (Wlaschek et al. 1988).

We propose that these discrepancies are most easily explained by the general tendency of cell cultures to eliminate heterochromatin. One of the terminal *Y*-chromosomal nucleolus organizers is lost from the *Y* chromosome of the KUN-DH-33 cell line. In addition, other heterochromatic regions of the genome have also been deleted. The heterochromatic arm of the *X* chromosome in KUN-DH-33 cells is considerably shorter compared with the wild type, and has lost its nucleolus organizer as well (Trapitz 1992, Trapitz et al. 1992). Also in *D. melanogaster* cell lines, karyotype polymorphisms frequently occur, including the loss of large portions of the *Y* chromosome (Dolfini 1971). Moreover, frequent transpositions such as have been reported to occur during the initial phases of establishing permanent *Drosophila* cell lines (Junakovic et al. 1988), might affect the structure of parts of the genome not under selective pressure. Therefore it is not certain whether sequences of *Y*-chromosomal male fertility genes that are not required for the survival of the cells are still present in the *Y* chromosome of a cell line, and if so, whether they have maintained their biologically active structure. Any data obtained from such systems must therefore be treated with appropriate reservations.

Concluding remarks

Our present knowledge, emerging from this work and from that of Trapitz et al. (1992), on the size and arrangement of the different types of repetitive DNA sequences in the region forming the lampbrush loop *Nooses*, can be summarized as follows: (i) in at least 300 kb of DNA, repeats of the ay1 family are organized in small tandem clusters interspersed with other DNA sequences, some of which are *Y* associated; (ii) for repeats of the YsI family such an interspersion has never been demonstrated; (iii) in addition, ay1 forms a large cluster of tandem ay1 repeats exceeding 800 kb of DNA; (iv) YsI is also present in a large cluster of tandem YsI repeats, which exceeds 600 kb; (v) the ay1 and YsI sequences are in separate locations on the short arm; (vi) at least some of the *Y*-associated sequences are transcribed in the *Nooses*. We propose that major parts of the loop are composed of co-transcribed ay1 repeats and sequences of the *Y*-associated type.

In the accompanying paper (Hochstenbach et al. 1993), we will address the question of the locations of the clusters of homogeneous ay1 and YsI repeats and of the transcribed *Y*-associated sequences on the short arm of the *D. hydei Y* chromosome.

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CHAPTER 3

Localization of the lampbrush loop pair *Nooses* on the *Y* chromosome of *Drosophila hydei* by fluorescence in situ hybridization

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Localization of the lampbrush loop pair *Nooses* on the *Y* chromosome of *Drosophila hydei* by fluorescence in situ hybridization

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Abstract. We have used fluorescence in situ hybridization to map the positions of the different repetitive DNA sequences from the region forming the lampbrush loop pair *Nooses* on the *Y* chromosome of *Drosophila hydei* This region harbours a megabase cluster of tandemly organized repeats of the *Y*-specific ay1 family and a megabase cluster of tandem repeats of the related *Y* specific YsI family In addition, ay1 repeats also occur in short blocks that are interspersed by other repetitive DNA sequences that we call *Y*-associated, since they have additional copies on other chromosomes Using specific probes for ay1, YsI and *Y*-associated DNA sequences, we show that there is one large proximal cluster of YsI repeats and one, more distally located, large cluster of ay1 repeats The *Y*-chromosomal copies of the *Y*-associated sequences are located in the most distal part of the ay1 cluster This is consistent with the juxtaposition of ay1 and *Y*-associated sequences in more than 300 kb of cloned genomic DNA Since both ay1 and *Y*-associated sequences have been shown to be transcribed in the *Nooses*, the lampbrush loop is formed in a distal region of the short arm of the *Y* chromosome, adjacent to the terminally located nucleolus organizer region The clusters of homogeneous ay1 and YsI repeats are of no functional significance for the formation of the lampbrush loop

Introduction

In the preceding paper (Hochstenbach et al 1993), we have described different types of clones containing repetitive DNA sequences from the region forming the lampbrush loop pair *Nooses* on the short arm of the *Y* chromosome of *Drosophila hydei* This loop is associated with the single male fertility gene *Q* on the short arm (Hackstein et al 1982, Hackstein 1987) More than 1000 kb of genomic DNA were recovered in more than 150 lambda and cosmid clones by screening genomic libraries with repeats of the *Y*-specific ay1 family, which is specifically transcribed in this lampbrush loop pair (Vogt et al 1982, Vogt and Hennig 1983) Three classes of sequence organization were identified (i) 300 kb of clones containing small clusters of ay1 repeats that are interspersed by a sequence type designated as *Y*-associated, since it has additional copies on other chromosomes (Vogt and Hennig 1983, 1986a), (ii) more than 300 kb of clones containing contiguous ay1 repeats without interspersion, and (iii) more than 400 kb of clones containing only repeats of the *Y*-specific YsI family YsI is an evolutionary derivative of ay1 (Wlaschek et al 1988), and has been claimed to be transcribed in the *Nooses* as well (Lifschytz et al 1983, Lifschytz and Hareven 1985, Trapitz et al 1992) Pulsed-field gel electrophoretic analysis indicated that the non interspersed ay1 repeats are organized in an 815 920 kb cluster of tandem repeats, and the YsI repeats are organized in a separate 630 710 kb cluster of tandem repeats (Trapitz et al 1992)

Thus, whereas the *Nooses*-forming region has a restricted sequence complexity, it is evident that the total amount of DNA containing ay1 and YsI repeats is much larger than the amount of DNA that is transcribed in the loop, which is a single transcription unit with a length not exceeding 260 kb (Grond et al 1983) Since we have shown that several of the *Y*-associated sequences are specifically transcribed in the *Nooses* (Vogt and Hennig 1986b, Hochstenbach et al 1993), we proposed as a working model that major parts of the loop have an organization similar to the genomic clones that contain both ay1 repeats and sequences of the *Y*-associated type (Vogt and Hennig 1986b, Hennig et al 1989, Hennig 1990)

In this paper we use two-colour fluorescence in situ hybridization on mitotic metaphase and on interphase chromosomes to investigate the organization of the ay1, YsI and *Y*-associated sequences within the *Y* chromosome We find that ay1 and YsI reside in separate domains, with YsI in a more proximal position on the short arm, close to the centromere The transcribed *Y*-asso-

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ciated sequences are organized in one single domain that overlaps with the distal part of the ay1 domain We conclude from these results that the chromosomal position of the lampbrush loop pair *Nooses*, and of male fertility gene *Q*, is defined by that of the ay1 repeats that are interspersed by co-transcribed *Y*-associated sequences The large clusters of homogeneous ay1 and Ys1 repeats are located outside of the *Nooses* transcription unit

Materials and methods

Fly strains D hydei individuals were taken from the Tübingen wild type strain from our laboratory collection Flies were kept at 18° or 24° C as described (Hochstenbach et al 1993)

Preparation of chromosome spreads Neuroblast metaphase and interphase preparations were obtained from male third instar larvae Brains were dissected in 0 95% (w v) sodium citrate, fixed in 3 1 ethanol acetic acid for at least 5 min, and transferred to a drop of 60% acetic acid on a slide warmer Three brains were used per slide After about 1 min cells were spread by tilting the slide and moving the drop over the entire slide Slides were stored at 4° C in 70% ethanol

Probes ay1 is defined as a *Y*-specific EcoRI DNA fragment of 393 bp (Vogt and Hennig 1986a) As a specific probe for detecting repeats of the ay1 family, we used clone PY9 (Vogt et al 1982, Vogt and Hennig 1983), containing a 9 kb PstI DNA fragment cloned in pBR322 It contains about 20 tandemly arranged ay1 repeats and produces the same hybridization pattern on Southern blots of genomic DNA from males as a single ay1 repeat (Vogt and Hennig 1986a)

As a probe specific for the ay1-related Ys1 family we used clone DhNo255, a 17 kb EcoRI DNA fragment cloned in λEMBL3 (Hochstenbach et al 1993) It contains a complex pattern of SalI fragments of 0 55–1 45 kb that all hybridize with PY9, but only under non stringent conditions This is a diagnostic parameter for the distinction of repeats of the Ys1 and the ay1 families (Wlaschek et al 1988) Sequencing of a SalI DNA fragment of 562 bp, and of larger DNA fragments as well, unambiguously identified the DhNo255 clone as belonging to the Ys1 family (unpublished data of R Hochstenbach, M Knops and W Hennig)

As a probe for detecting transcribed *Y*-associated sequences, we used the 5 8 kb BamHI-EcoRI DNA fragment from clone DhNo90 (designated as DhNo90BE5 8, see Hochstenbach et al 1993), that was subcloned in pGEM3 (Promega)

Probes were labelled by nick translation either with digoxigenin-11 dUTP (Boehringer Mannheim) or biotin-14-dA I P (Gibco BRL Life Technologies), according to the manufacturer's protocol The labelled DNAs were purified using Sephadex G50 spin columns, precipitated and dissolved at concentrations of 2–3 ng µl in a hybridization buffer consisting of 50% (v/v) deionized formamide (Fluka), 2 × SSC, 10% (w v) dextran sulphate, 1% (v/v) Tween-20, and 0 5 µg/µl sonicated herring sperm DNA 1 × SSC is 0 15 M NaCl 0 015 M sodium citrate, with pH adjusted at 7 2 Probes were denatured by incubation at 80° C for 10 min

In situ hybridization Slides were air dried and incubated for 1 min in 70% acetic acid at room temperature (RT), quickly rinsed in PBS (3 mM KCl, 1 5 mM KH_2PO_4, 137 mM NaCl, 7 mM Na_2HPO_4, with pH adjusted at 7 4) and washed three times for 5 min each in PBS, all at RT Then they were passed through a series of 70%, 90%, 96% and 100% ethanol and air dried

For removal of endogenous RNA, slides were incubated for 60 min at 37° C in 2 × SSC containing 100 µg/ml pre-boiled RNase A, washed three times for 5 min each in 2 × SSC at RT, passed through the ethanol series and air dried

Chromosomal DNA was denatured by incubating the slides for precisely 2 min at 70° C in 70% (v v) deionized formamide in 2 × SSC Then the slides were immediately washed in ice-cold 2 × SSC (three washes of 2 min each) passed through the ethanol series and air dried For hybridization, 24 µl of hybridization buffer, containing the appropriate combination of denatured probes, was applied on each slide and sealed with a 24 × 50 mm coverslip Slides were incubated overnight at 37° or 50° C in a moist chamber depending on the stringency required

Probe detection by immunofluorescence After hybridization, slides were washed three times for 5 min each in 50% (v/v) formamide in 2 × × SSC at 42° C, followed by three washes in 2 × SSC at the same temperature They were subsequently washed in Wash Buffer 1 [WB1 4 × SSC containing 0 05% (v/v) Tween-20, pH 7 0] for 3 min at RT and pre incubated for 10 min at RT in Block Buffer 1 [BB1, WB1 containing 5% (w/v) Protifar non-fat dry milk powder (Nutricia, Zoetermeer The Netherlands)] For this, and all subsequent immunological (pre)incubation steps, a volume of 100 µl was pipetted onto the slide, sealed with a 24 × 50 mm coverslip, and slides were placed in a moist chamber at the appropriate temperature

After blocking slides were incubated for 20 min at RT in BB1 containing avidin-FITC (fluorescein isothiocyanate, Vector Laboratories Burlingame, USA, 1 500 dilution of a 5 mg/ml stock solution) Slides were washed in WB1 three times for 5 min each at RT, followed by washing for 5 min at RT in Wash Buffer 2 [WB2, 0 1 M Na_2HPO_4, 0 1 M NaH_2PO_4, 0 05% (v/v) Nonidet P-40, pH 8 0]

Then slides were incubated for 30 min at 37° C in Block Buffer 2 [BB2 WB2 containing 5% (w v) non-fat dry milk powder] with mouse antidigoxin monoclonal antibodies (Sigma, St Louis USA, 1 200 dilution) and biotinylated goat anti-avidin antibodies (Vector Laboratories, 1 200 dilution) Slides were subsequently washed three times in WB2 for 5 min each at RT

As a following step, slides were incubated for 30 min at 37° C in BB2 containing digoxigenin conjugated sheep anti-mouse antibodies (Boehringer Mannheim, 1 100 dilution) and avidin-FITC (1 500 dilution), and then washed in WB2 three times for 5 min each at RT

Subsequently, slides were incubated for 30 min at 37° C in BB2 containing rhodamine-conjugated sheep anti-digoxigenin antibodies (Boehringer Mannheim 1 20 dilution) and biotinylated goat anti-avidin antibodies (1 200 dilution) and washed three times for 5 min each in WB2 at RT

Finally, slides were incubated for 30 min at 37° C in BB2 containing Texas Red-conjugated rabbit anti sheep antibodies (Jackson Immunoresearch Laboratories, West Grove, USA, 1 100 dilution) and avidin-FITC (1 500 dilution) washed once in WB2 and twice in PBS all for 5 min each at RT, dehydrated by passage through the ethanol series, and air dried

The air-dried slides were mounted in an anti-fade solution (9 parts glycerol and 1 part 0 2 M Tris-HCl, pH 8 0) containing 2 3% (w v) DABCO (1 4-diazobicyclo-(2 2,2)-octane, Merck, Darmstadt, Germany) To this solution DAPI (4 ,6-diamidino-2-phenylindole dichloride, Sigma) was added to a final concentration of 0 5 µg/ml for blue counterstaining of chromosomes and chromatin Mounted slides were stored at 4° C

Fluorescence microscopy and image analysis Preparations were studied under a Zeiss Axiophot epifluorescence microscope, equipped with appropriate filters for separate visualization of DAPI (487701) Rohdamine Texas Red (487715) and FITC (487709 and 447710) fluorescence, as well as for simultaneous visualization of Texas Red and FITC fluorescence (Omega double small-band pass filter, Omega Corporation Brattleboro, USA) For photography, separate digital images for DAPI, Texas Red and FITC, respectively, were recorded using a Photometrics high-performance CH250 A cooled CCD-camera (Photometrics, Tucson, USA), which was coupled to a Macintosh IIci computer The three images were superimposed and displayed in blue, red, and

green pseudocolours on the monitor using a modified version (Biological Detection Systems Pittsburgh USA) of the image analysis and processing program TCL-image (TNO Institute of Applied Physics Delft The Netherlands) Photographs were made directly from the monitor on Kodak EPP 100 plus colorslide film using a Polaroid Quickprint Video Print camera system

Results

The experiments described in this paper were designed to determine the locations of the interspersed ay1 repeats and those of the homogeneous ay1 and Ysl repeat blocks on the short arm of the *D hydei Y* chromosome The length of this chromosome is about 43000 kb (Zacharias et al 1982) and as estimated from metaphase plates, the short arm contains not more than 6000 kb of DNA For the mapping of DNA sequences on such a small cytological target, we used two-colour fluorescence in situ hybridization methodology, allowing the simultaneous visualization of the chromosomal positions of two different sequences In addition, we employed interphase chromatin mapping, using G2 nuclei in which the chromatin is much less condensed compared with metaphase chromosomes (Lawrence et al 1988 1990, Trask et al 1989) The relative order of three closely linked probes, which cannot be resolved at the level of the metaphase chromosome can be directly determined in interphase chromatin if the centrally located DNA sequence is detected by a fluorochrome different from that of the two flanking DNA sequences (Lawrence et al 1990, Trask et al 1991)

Using PY9 and DhNo255 as family-specific DNA probes for ay1 and Ysl, respectively, we first attempted to map the relative positions of the ay1 and Ysl clusters on metaphase chromosomes When used separately, ay1 and Ysl probes both hybridize to a central position on the short arm (Vogt et al 1986, Vogt and Hennig 1983, Lifschytz et al 1983, Lifschytz and Hareven 1985, Wlaschek et al 1988) After simultaneous hybridization of both probes we found that both signals co-localize at one position centrally on the short arm (Fig 1) In interphase chromosomes identical results were obtained at the stringent and non-stringent hybridization temperatures Although the Y chromosome is heterochromatic and therefore more condensed during interphase than the euchromatin, each probe maps to a separate position in interphase nuclei (Fig 2) This is consistent with the molecular data of Trapitz et al (1992), demonstrating the existence of separate clusters of ay1 and Ysl Since in interphase chromatin these clusters are clearly separated from each other, other *Y*-chromosomal DNA sequences must be located in between In a portion of the ay1 cluster more distant from the Ysl cluster, the ay1 signal becomes weaker than in the other portion (Fig 2, right nucleus) This may reflect differential chromatin condensation within the ay1 cluster but, more likely, it is a consequence of the interspersion of ay1 repeats in this portion by other sequences (see below) The clusters are located in close proximity to the cytologically visible nucleolus This is not unexpected since both distal ends of the *Y* chromosome carry a nucleolus organizer region (NOR) (Hennig et al 1975)

As a next step, we performed simultaneous hybridization of the ay1-specific PY9 probe and the DhNo90BE5 8 probe The latter specifically detects transcribed *Y*-associated sequences of the *Nooses* lampbrush loop pair (Hochstenbach et al 1993) Identical results were obtained at stringent and non-stringent hybridization temperatures On metaphase chromosomes the *Y*-associated probe hybridized to one position on both the *Y* and the *X* chromosome (Fig 3) Each of these signals can be seen as two laterally adjacent dots, representing the two chromatids The signal on the *X* chromosome is located in the proximal part of the euchromatic arm A single pair of dots is also found in interphase chromatin (Fig 4) This and the strength of this signal indicate that multiple copies are located close to one another

The signal of the *Y*-associated probe on the metaphase *Y* chromosome overlaps with the distal portion of the more extended ay1 signal (Fig 3) This is expected from the interspersion of both sequence types in several genomic clones (Hochstenbach et al 1993) Again, the strength of the signal indicates the presence of multiple copies of the *Y*-associated sequence Also these must be closely linked, since in interphase nuclei the signal of the *Y*-associated probe consists of a single pair of dots (Fig 4) As noticed earlier, the ay1 signal is weaker on one side of the ay1 cluster (Fig 2) The position of the weaker signal overlaps with that of the *Y*-associated

Fig 1 Simultaneous detection of the ay1 specific PY9 and the Ysl specific DhNo255 DNA probes in neuroblast metaphase chromosomes by fluorescence in situ hybridization The ay1 probe was labelled with biotin and detected with fluorescein isothiocyanate (FITC) (pseudocoloured *green*) and the Ysl probe was labelled with digoxigenin and detected with Texas Red (pseudocoloured *red*) On this and on the other photographs the green pseudocolour appears *light blue* and when overlapping with the *red* pseudocolour it appears *white* Chromosomes were counterstained with 4 6-diamidino 2 phenylindole (DAPI) (pseudocoloured *blue*) X and *Y* chromosomes are indicated The long arm of the *Y* chromosome has a constriction (Bonaccorsi et al 1981) which is a useful marker for distinguishing the long arm (indicated by an *arrow*) Both probes overlap at a central position on the short arm of the Y chromosome *Bar* represents 5 μm

Fig 2 Simultaneous detection of ay1 and Ysl in neuroblast interphase nuclei by fluorescence in situ hybridization Labelling of probes and pseudocolouring were as in Fig 1 Each probe hybridizes to a separate cluster *Bar* represents 5 μm

Fig. 3. Simultaneous detection of the ay1-specific PY9 and the *Y* associated DhNo90BE5 8 DNA probes in neuroblast metaphase chromosomes by fluorescence in situ hybridization The ay1 probe was labelled with biotin and detected with FITC (pseudocoloured *green*) the *Y* associated probe was labelled with digoxigenin and detected with Texas Red (pseudocoloured *red*) Chromosomes were counterstained blue with DAPI (pseudocoloured *blue*) *X* and Y chromosomes are indicated the *arrow* pointing to the constriction in the long arm of the Y chromosome The signal of the *Y*-associated probe overlaps with the distal portion of the ay1 signal on the short arm of the *Y* chromosome Additional copies of the *Y*-associated sequence are in the proximal part of the euchromatic arm of the *Y* chromosome *Bar* represents 5 μm

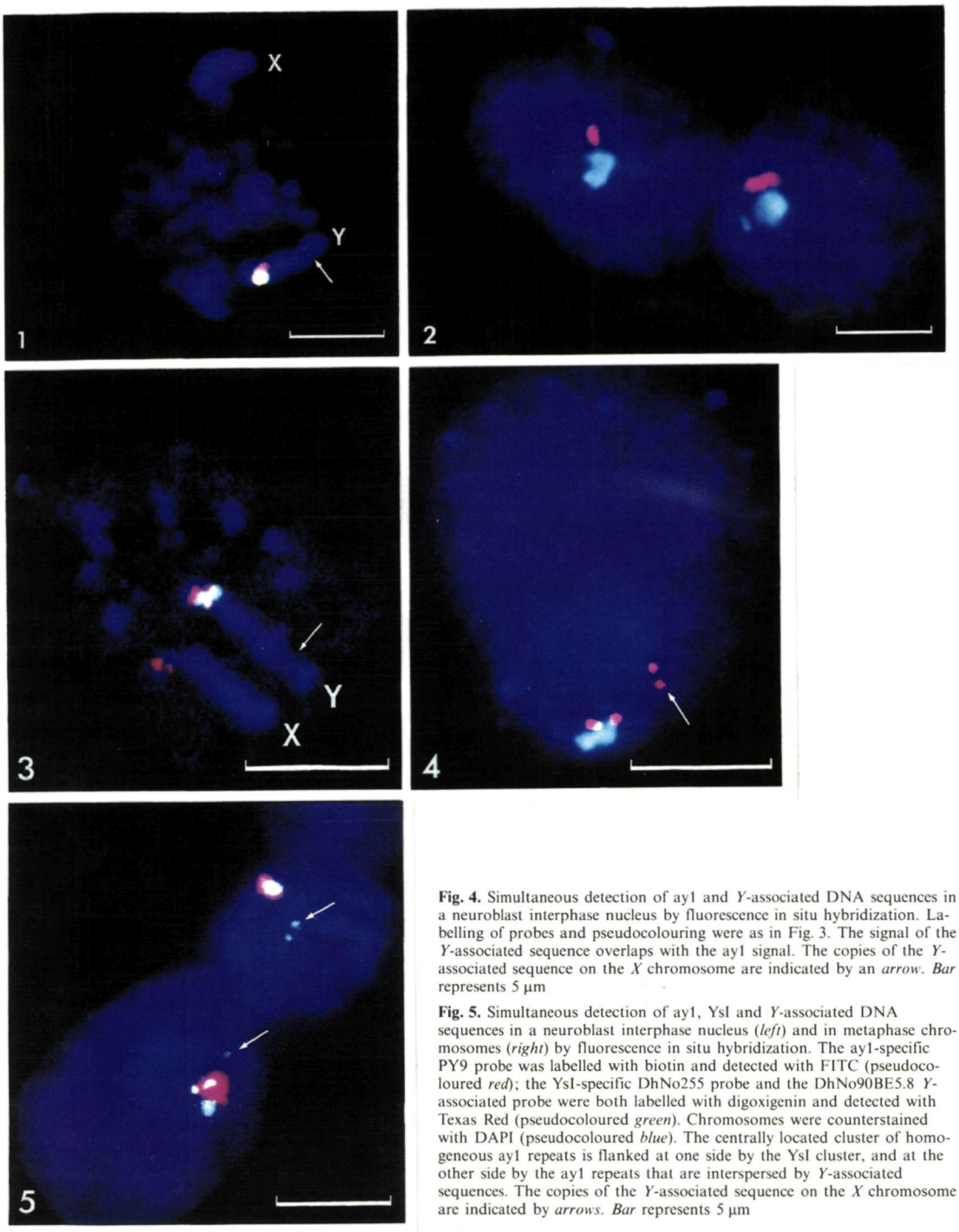

Fig. 4. Simultaneous detection of ay1 and *Y*-associated DNA sequences in a neuroblast interphase nucleus by fluorescence in situ hybridization. Labelling of probes and pseudocolouring were as in Fig. 3. The signal of the *Y*-associated sequence overlaps with the ay1 signal. The copies of the *Y*-associated sequence on the *X* chromosome are indicated by an *arrow*. *Bar* represents 5 μm

Fig. 5. Simultaneous detection of ay1, YsI and *Y*-associated DNA sequences in a neuroblast interphase nucleus (*left*) and in metaphase chromosomes (*right*) by fluorescence in situ hybridization. The ay1-specific PY9 probe was labelled with biotin and detected with FITC (pseudocoloured *red*); the YsI-specific DhNo255 probe and the DhNo90BE5.8 *Y*-associated probe were both labelled with digoxigenin and detected with Texas Red (pseudocoloured *green*). Chromosomes were counterstained with DAPI (pseudocoloured *blue*). The centrally located cluster of homogeneous ay1 repeats is flanked at one side by the YsI cluster, and at the other side by the ay1 repeats that are interspersed by *Y*-associated sequences. The copies of the *Y*-associated sequence on the *X* chromosome are indicated by *arrows*. *Bar* represents 5 μm

signal, demonstrating the subdivision of the ay1 cluster into a region with uninterrupted ay1 repeats (the stronger signal close to the YsI cluster), and a region with interspersed ay1 repeats (the weaker signal further away from the YsI cluster) The separated dot-like signals of the *Y*-associated DNA probe in interphase nuclei indicate the separation of the chromatids towards the distal end of the short arm This may be of functional significance, since the more distally located ribosomal genes are probably transcriptionally active, as implied by the close proximity of the *Y*-specific signals to the nucleolus Also the *X*-chromosomal signals of the *Y*-associated sequence are close to the nucleolus, which is expected as there is a NOR in the distal *X* heterochromatin (Meyer and Hennig 1974 Hennig et al 1975) Thus, *X* and *Y*-chromosomal NORs are probably transcriptionally active in mitotic interphase cells (also see Bonaccorsi et al 1981)

For an unambiguous determination of the relative order of the signals obtained by the ay1, YsI and *Y*-associated DNA probes on the short arm of the *Y* chromosome, we performed a two-colour three-probe hybridization on interphase chromatin It emerged that the uninterrupted ay1 repeat cluster occupied a central position, being flanked by the YsI cluster at one side and by the ay1 repeats interspersed with *Y*-associated sequences at the other side (Fig 5) Together with the metaphase mapping results and with the rDNA localization by Hennig et al (1975) this implies that the order of repetitive DNA sequences on the short arm of the *Y* chromosome is centromere YsI cluster homogeneous ay1 repeats interspersed ay1 repeats rDNA telomere

Discussion

Sensitivity and fidelity of the in situ hybridization method

We have localized three classes of repetitive DNA sequences that we identified as constituents of the region forming the lampbrush loop pair *Nooses* on the short arm of the *D hydei Y* chromosome The probes used reflect the rather restricted sequence complexity of this region (Hochstenbach et al 1993) PY9 and DhNo255 have earlier been shown to be specific probes for the ay1 and YsI families, respectively (Vogt and Hennig 1986a, Hochstenbach et al 1993) Cross-hybridization between repeats of these two related families does not occur in our experiments The DhNo90BE5 8 probe is assumed to be representative of the *Y*-associated sequences in the *Nooses* region for the following reasons Firstly, it has at least ten copies on the *Y* chromosome Secondly, it is immediately adjacent to ay1 repeats in four different genomic DNA clones Thirdly, it cross-hybridizes with other *Y*-associated sequences in at least three additional DNA clones (Hochstenbach et al 1993) Together, these seven clones represent a total of 137 kb of DNA Fourthly, since this sequence is specifically transcribed in the *Nooses* lampbrush loop pair (Hochstenbach et al 1993), it is most likely representative of the transcribed *Y*-associated DNA of the loop

The length of the short arm of the *Y* chromosome is about 6000 kb or less, carrying a central region accommodating the *Nooses*, as identified by metaphase mapping of ay1 repeats that are specifically transcribed in this loop pair (Vogt and Hennig 1983) The size of this region is close to the 2000–3000 kb resolution limit of metaphase mapping, as defined by the minimal distance of sequences that can be resolved along the length of the chromosome (Lawrence et al 1990) Therefore, the failure to see the separate clusters of ay1 and YsI repeats at this level, can be explained by the size of the genomic targets formed by these clusters, which are about 1 Mb (Trapitz et al 1992), and by a possible distortion of the linear order of the sequences in the condensed metaphase chromosomes (Trask et al 1991) However, using the Hoechst 33258 bands described by Bonaccorsi et al (1981) as a reference, K H Glatzer (cited by Trapitz et al 1992) has been able to localize YsI at a more proximal position than ay1 on the metaphase *Y* chromosome The clear separation of ay1 and YsI signals in interphase nuclei (Figs 2 5), illustrates the 50- to 100-fold greater resolving power of interphase chromatin mapping, which has a resolution limit of 25–50 kb (Lawrence et al 1990, Trask et al 1991)

The three-dimensional arrangement of chromosomes in interphase nuclei is generally accepted to be faithfully represented in fixed two-dimensional preparations, and chromosomal regions harbouring ribosomal genes are always in close association with the nucleolus (Manuelidis and Borden 1988, Emmerich et al 1989) This is also observed here for the short arm of the *Y* chromosome and the *X* heterochromatin Further, our interphase mapping experiments reveal an intriguing property of heterochromatin Since the large ay1 and YsI blocks are less condensed during interphase compared with metaphase it is clear that heterochromatin is not always maximally condensed during interphase of somatic tissues As discussed by Hennig (1985), the functional significance of this phenomenon is unclear

Organization of ay1 and YsI repeats in separate clusters

Our combined metaphase and interphase mapping data document that each family or repetitive DNA sequences is localized in a separate domain on the short arm Interphase mapping indicates that the homogeneous YsI repeats are separated from the ay1 repeats by other, unknown sequences that are neither homologous to ay1, nor to the *Y*-associated DhNo90BE5 8 probe Since there is only one ay1 cluster, the homogeneous ay1 repeats and the interspersed ay1 repeats are not separated by detectable amounts of other sequences The total size of the ay1 cluster is therefore given by the combined amount of the homogeneous ay1 repeats, which contribute 815–920 kb (Trapitz et al 1992) and of the interspersed ay1 repeats, contributing at least 300 kb (Hochstenbach et al 1993) The size of the ay1 cluster thus exceeds 1 Mb

Also molecular analyses imply that ay1 and YsI repeats are organized in separate blocks (Wlasckek et al

1988, Trapitz et al 1992, Hochstenbach et al 1993) It is therefore very unlikely that the ay1 cluster contains small amounts of Ys1 repeats (or vice versa) that would not be detected by in situ hybridization

Organization of homogeneous and interspersed ay1 repeats and of Y-associated sequences

In more than 300 kb of cloned genomic DNA, ay1 repeats are interspersed by *Y*-associated sequences (Hochstenbach et al 1993) Consistently, we find that the signal from the *Y*-associated DhNo90BE5 8 sequence overlaps with that of ay1 Moreover, the ay1 repeats that are interspersed by this sequence must be clustered together distally from the homogeneous ay1 repeats, since even in interphase chromatin, only one signal of the *Y*-associated sequence is seen at this position The fact that in the distal portion of the ay1 cluster the ay1 signal is detectably weaker than in the other portion, indicates that the distally located ay1 repeats are interspersed by a substantial amount of other DNA sequences Indeed, as judged from restriction maps ay1-containing genomic clones from this region contain at least one-third of other DNA sequences (Hochstenbach et al 1993) From Southern blots of genomic DNA we have no evidence for tandem repetition of DhNo90BE5 8 in the genome (Hochstenbach et al 1993) and therefore most, if not all, *Y*-chromosomal copies of this *Y*-associated sequence must be located between ay1 repeat clusters This is also true for the *Y*-associated sequences that cross-hybridize to DhNo90BE5 8, since at less stringent hybridization conditions, there is only one *Y*-chromosomal DhNo90BE5 8 signal even in interphase nuclei Although we cannot exclude that other *Y*-associated sequences are located in the proximal part of the ay1 cluster, we regard this as a highly unlikely possibility Trapitz et al (1992) have demonstrated the existence of at least 815 kb of uninterrupted ay1 repeats They can only be located in the proximal part of the ay1 cluster

The hybridization of DhNo90BE5 8 to the proximal part of the euchromatic arm of the *X* chromosome is consistent with hybridization on polytene chromosomes, which also shows a strong signal at this position (Hochstenbach et al 1993) Additional hybridization to polytene chromosome 6 is not reflected in metaphase chromosome hybridization The failure to detect copies on this small chromosome indicates that they are less numerous compared with those on the *X* chromosome In polytene chromosomes they may be polytenized and therefore more readily detected It is striking that the *X*-chromosomal signal of DhNo90BE5 8 is restricted to a small region of the chromosome, even in interphase nuclei On the *X* chromosome however, the copies of this sequence must have an organization different from that of the copies on the *Y* chromosome, since ay1 repeats are not found on the *X* (Vogt and Hennig 1983, 1986a)

Localization of the lampbrush loop pair Nooses on the short arm of the Y chromosome

The question of the localization of the *Nooses* lampbrush loop, and of the male fertility gene *Q*, can now be answered All evidence for the chromosomal position of the lampbrush loop has so far relied exclusively on the hybridization of cloned repetitive sequences from the short arm to loop transcripts For example, Trapitz et al (1992) have suggested that the loop is composed of uninterrupted ay1 and Ys1 repeats However, on the basis of their physical sizes it can be excluded that these large clusters are transcribed in their entirety in the 260 kb long lampbrush loop Because both ay1 and the DhNo90BE5 8 *Y*-associated sequence are specifically transcribed in the *Nooses* (Hochstenbach et al 1993), and give identical signals after simultaneous hybridization on *Nooses* transcripts (R Hochstenbach, R Suijkerbuijk and W Hennig, unpublished data), the chromosomal position of the *Nooses* transcription unit is defined by that of the *Y*-chromosomal copies of the DhNo90BE5 8 sequence The lampbrush loop is therefore located at the distal end of the megabase-sized ay1 cluster, adjacent to the more distally located nucleolus organizer, and only the distal part of the ay1 cluster is transcribed

Other observations are consistent with this conclusion Firstly, probes containing Ys1 repeats do not hybridize to *Nooses* transcripts under stringent conditions (R Hochstenbach M Knops and W Hennig, unpublished data) Secondly, the phylogenetically more ancient species *D eohydei* has few or no Ys1 repeats in its genome, but the co-transcription of ay1 and DhNo90BE5 8 in one *Y*-chromosomal lampbrush loop is conserved in this species (R Hochstenbach R Suijkerbuijk and W Hennig, unpublished data) Taken together all these observations imply that the homogeneous ay1 and Ys1 clusters are without functional relevance for lampbrush loop formation by male fertility gene *Q*

The *Nooses* are not the only loop pair of *D hydei* containing *Y*-associated sequences We have shown earlier that the loop pairs *Threads* and *Pseudonucleolus* also contain *Y*-associated sequences (Huijser et al 1988) The interspersion of rapidly evolving, loop-specific repetitive DNA sequences and more conserved, *Y*-associated sequences therefore seems to be a general feature of the lampbrush loops formed by the male fertility genes on the *Y* chromosome of *Drosophila* The transcribed repeats may be simple pentameric satellite DNA sequences that are not *Y*-specific, as in *D melanogaster* (Bonaccorsi et al 1990, reviewed by Gatti and Pimpinelli 1992), or they may be more complex as in *D hydei* and, with few exceptions, specific for the *Y* chromosome (reviewed by Hennig et al 1989 Hennig 1990) *Y*-associated sequences of *Threads* and *Pseudonucleolus* (Huijser et al 1988) have been identified as defective retrotransposons of the *micropia* family Those of the *Nooses* are defective retrotransposons of the *gypsi* family, and DhNo90BE5 8 belongs to this family (R Hochstenbach, H Harhangi, K Schouren and W Hennig in preparation) It is at present unknown whether the transcribed

nple satellite repeats in the lampbrush loops of *D.* *lanogaster* are also interspersed by (defective) transsable elements.

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CHAPTER 4

Discrimination of transcribed and non-transcribed related repetitive DNA sequences from the *Y* chromosomes of *Drosophila hydei* and *Drosophila eohydei*

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Abstract. The short arm of the *Y* chromosome of *Drosophila hydei* carries a single male fertility gene, gene *Q*, which forms the lampbrush loop pair *Nooses*. Conflicting observations have been reported concerning the identity of the of repetitive DNA sequences that are transcribed in this loop pair. It has been claimed by other investigators that the loop transcripts contain repeats of two distinct, but related families of *Y*-specific repetitive DNA sequences, ayl and YsI. We reinvestigated this issue, using as probes single ayl and YsI repeats which, under stringent conditions, hybridize only to members of their own family. Under non-stringent conditions, both repeats hybridize *in situ* to *Nooses* transcripts. However, if hybridization conditions are stringent, only the ayl probe hybridizes to loop transcripts. Hybridizations to Northern blots of testis RNA confirm these results. Further, YsI repeats are not found in the closely related species *D. eohydei*. We conclude that the YsI repeats are not relevant for the function of fertility gene *Q*.

Introduction

The *Y* chromosome of *Drosophila* does not carry genes required for viability and bears relatively few other genetic functions (reviewed by Hackstein 1987; Hennig 1990; Gatti and Pimpinelli 1992). This heterochromatic chromosome consists largely, if not exclusively, of repetitive DNA sequences, both in *D. hydei* (Hennig 1968; Hennig et al. 1974b), and in *D. melanogaster* (Bonaccorsi and Lohe 1991). At the molecular level, the *Y* chromosome of *D. hydei* has been studied most intensively. It carries at least seven genes that are required for male fertility, five of which form prominent lampbrush loop pairs during meiotic prophase (Hackstein et al. 1982; Hackstein 1987). These loops are giant transcription units with sizes between 260 kb and 1000 kb or more (Grond et al. 1983; de Loos et al. 1984). They seem to contain only repetitive DNA sequences that do not encode proteins (reviewed by Hennig 1985, 1990; Hennig et al. 1989).

In order to understand how the lampbrush loop-forming genes contribute to male fertility, we have decided to reconstruct an entire loop in an ordered set of overlapping clones. We have focused on the smallest loop pair of *D. hydei*, the *Nooses*, formed by fertility gene *Q* on the short arm of the *Y* chromosome (Hackstein et al. 1982). Miller spreading experiments have indicated that the loop is a single transcription unit with a length of about 260 kb (Grond et al. 1983).

We have previously shown that the transcripts of the *Nooses* loop pair contain two major types of repetitive DNA sequences: repeats of the *Y*-specific ayl family (Vogt et al. 1982; Vogt and Hennig 1983,1986a) and defective retrotransposons of the *gypsy* family (Chapter 5). We have isolated several genomic clones that contain both types of sequences. Therefore, these clones represent potential segments of the transcription unit (see Chapters 2 and 5). In addition, several other investigators have claimed that repeats of the ayl-related, *Y*-specific YsI family of repetitive DNA sequences are also transcribed in the loop (Lifschytz et al. 1983; Lifschytz and Hareven 1985; Trapitz et al. 1992). YsI is an evolutionary derivative of the more ancient ayl family (Wlaschek et al. 1988).

There are, however, reasons to suspect that the hybridization of YsI repeats to loop transcripts represents an experimental artefact, since repeats of both families cross-hybridize under conditions of moderate stringency (Wlaschek et al. 1988; Chapter 2). Repeats of the YsI family have 65-75% nucleotide similarity with repeats of the ayI family, whereas repeats of the same family share 80-90% similarity.

Moreover, the short arm of the *Y* chromosome carries considerably more DNA containing ayI and YsI repeats than can be accommodated within the 260 kb loop length as determined by Miller spreading. By pulsed-field gel electrophoresis of DNA from a cell line carrying the *Y* chromosome, Trapitz et al. (1992) have shown that ayI and YsI repeats are organized in separate clusters of tandem repeats, that have sizes of at least 815 and 630 kb, respectively. In addition, using ayI repeats as a probe, we have cloned 400 kb of DNA containing uninterrupted YsI repeats, 300 kb of DNA containing uninterrupted ayI repeats and another 300 kb of DNA consisting of ayI repeats that are interspersed with unrelated repetitive DNA sequences, such as defective retrotransposons of the *gypsy* family (see Chapters 2, 5 and 7). By *in situ* hybridization to metaphase chromosomes we have localized the large clusters of ayI and YsI repeats at non-overlapping positions on the short arm of the *Y* chromosome, with that of YsI being more proximal than that of ayI (Chapter 3). Since the transcribed *gypsy* sequences are located in the most distal part of the ayI cluster, the position of the YsI repeats does not overlap with that of the *Nooses* loop pair.

In this Chapter, we use Northern blot and transcript *in situ* hybridization to reinvestigate the question of whether repeats of the YsI family are transcribed in the *Nooses* lampbrush loop pair. We use single repeats of the ayI and YsI families as probes, which under stringent conditions hybridize exclusively with repeats of their own family. We find that, consistent with our other findings (Chapters 2 and 3), YsI repeats are not transcribed in the *Nooses* lampbrush loop.

Materials and methods

Drosophila strains. Both the *D. hydei* wild-type strain *Tübingen* and the *D. eohydei* wild-type strain, that were used throughout this study, were from our laboratory collection. As a control, we used *X/ms(Y)Q1* males carrying a deletion of the short arm of the *D. hydei Y* chromosome, and therefore missing fertility gene *Q* and the associated loop pair *Nooses* (Hackstein et al. 1982; Hackstein and Hennig 1982). (The *ms(Y)Q1 Y* chromosome is known as *GE7*). After its induction in 1979, *ms(Y)Q1* originally had a short arm carrying an ethyl methylsulphonate- induced male-sterile allele of gene *Q* that forms a visible *Nooses* lampbrush loop pair. However, during maintenance of the stock, the short arm was lost (J.H.P. Hackstein, personal communication). Absence of the short arm was confirmed by inspecting brain metaphase preparations of *X/ms(Y)Q1* third instar larvae and by the failure to detect ayI repeats, which are specifically located on the short arm (Vogt and Hennig 1983), in Southern blots of genomic DNA prepared from such males (not shown). The *X/ms(Y)Q1* males were obtained as described (Chapter 2).

Flies were maintained at 18°C on a medium containing dried yeast, cornmeal, soy flour, malt and sugar-beet syrup, that was inoculated with live baker's yeast.

Nucleic acid probes. The sequence complexity of the members of the ay1 family of repetitive DNA sequences is represented by a 393 bp EcoRI DNA fragment, named ay1 (Vogt and Hennig 1986a). We used this fragment as a specific probe for the ay1 family. The basic repeat of the YsI family is a 550-600 bp SalI DNA fragment (Lifschytz and Hareven 1985; Wlaschek et al. 1988). As a specific probe for the YsI family, we used a 562 bp SalI DNA fragment, subcloned from lambda clone DhNo255. It was isolated from an EcoRI genomic library that was screened under non-stringent conditions, using ay1 sequences as a probe (see Chapter 2). Both the ay1 EcoRI fragment and the YsI SalI fragment were subcloned in pBluescriptII KS+ plasmid vectors (Stratagene). Since ay1 and YsI probes hybridize strand-specifically with *Nooses* transcripts, we determined the orientation of the insert relative to the T3 and T7 polymerase promoters, following conventional DNA sequencing protocols (Sambrook et al. 1989).

Isolation of nucleic acids. DNA was extracted from young adult flies as described by Huijser and Hennig (1987). For the isolation of RNA, 3 to 5 day-old males were used. Extraction of total RNA from testes and carcass (i.e. the remaining parts of the males after dissection of gonads) was according to the method of Chirgwin et al. (1979) as described by Brand and Hennig (1989). For separation of polyadenylated and non-polyadenylated RNA, the mRNA purification kit of Pharmacia was used following Pharmacia protocols. Plasmid DNA was isolated following a boiling procedure provided by Stratagene.

Labelling of probes. Linearized plasmid DNAs were purified by phenol/chloroform extraction and ethanol precipitation, and used for producing strand-specific RNA probes by *in vitro* transcription using either T3 or T7 polymerase (Stratagene) according to Boehringer Mannheim protocols. Probes for *in situ* hybridization were labelled by incorporation of digoxigenin-11-UTP (DIG; Boehringer Mannheim). Probes for hybridization to Northern blots were labelled by incorporation of [^{32}P]-UTP. For hybridization to Southern blots of genomic DNA, as well as for some Northern blots, purified ay1 or YsI insert DNA was labelled by nick translation using [^{32}P]-dCTP (Sambrook et al. 1989).

Hybridization on Southern and Northern blots. For Southern blots of genomic DNA from males, DNA was digested with restriction enzymes, and fragments were separated on 0.45% or 0.8% agarose gels. Approximately 3 µg DNA was loaded in each lane. Fragments were immobilized on Hybond-N nylon membranes (Amersham). Membranes were hybridized and washed as described in Chapter 2. Non-stringent conditions corresponded to post-hybridization washes in 2 x SSC/0.1 % SDS at 65°C, stringent conditions to washes in 0.1 x SSC at 65°C. 1 x SSC is 0.15 M NaCl, 0.015 M sodium-citrate, with pH adjusted to 7.2.

For Northern blots, glyoxal/dimethylsulfoxide-denatured RNA was separated on 1% or 2% agarose gels, using RNA ladders from BRL as size markers. Approximately 20 µg total RNA was loaded in each lane. RNA was blotted onto Gene Screen Plus membranes and hybridized as described by Brand and Hennig (1989). RNA blots were successively washed in 0.3 M Na_2HPO_4 (pH 7.2)/1% (w/v) SDS, in 0.1 M Na_2HPO_4 (pH 7.2)/0.5% (w/v) SDS, and in 0.02 M Na_2HPO_4 (pH 7.2), all at 50°C.

Non-radioactive in situ hybridization. For hybridization of ay1 and YsI probes to lampbrush loop transcripts *in situ*, we we used the protocol of Tautz and Pfeifle (1989), as described in Chapter 2. The protocol is based on the detection of DIG-labelled RNA-RNA hybrid molecules with an anti-DIG antibody that is conjugated with alkaline phosphatase (Boehringer Mannheim). In some experiments the hybridizations were performed in 50% formamide/6 x SSC at 50°C, following the original protocol. In other experiments the probes were hybridized in 2 x SSC at temperatures varying from 65°C to 80°C.

Results

Specific hybridization of ayl and YsI probes to repeats of their own family

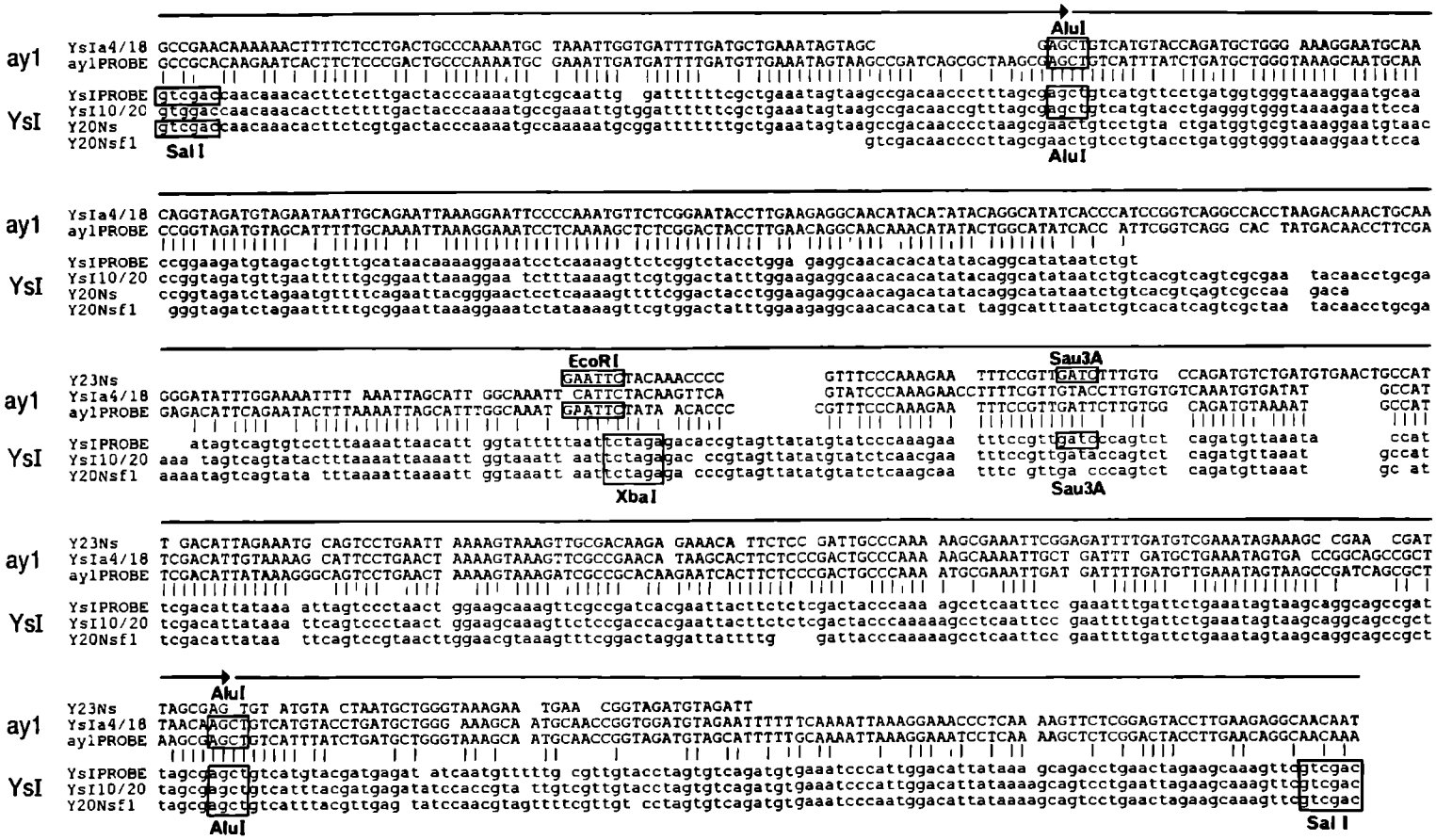
Throughout all the experiments reported in this paper we used single ayl and YsI repeats as a probe. The sequences of these repeats are shown in Fig. 1, where they are aligned with the ayl and YsI repeats that were used as a probe by other investigators (Lifschytz and Hareven 1985; Lifschytz 1987; Wlaschek et al. 1988; Trapitz et al. 1988). The ayl and YsI repeats used in this study have 70-75% sequence similarity to repeats of the other family, but 75-90% sequence similarity to repeats of their own family (Table 1; also see Chapter 6).

We used SalI digests of genomic DNA from males to test the specificity of the ayl and YsI probes (Fig. 2). This enzyme has conserved cleavage sites in YsI repeats but not in ayl repeats (Wlaschek et al. 1988; Trapitz et al. 1992; Chapter 2). Therefore, all ayl repeats are present in large SalI DNA fragments that are not resolved in normal agarose gels, whereas YsI repeats are cleaved into SalI DNA fragments with a basic repeat length of 550-600 bp. As expected, each probe hybridizes to both ayl and YsI repeats under non-stringent conditions (Fig. 2A). However, under stringent conditions, the ayl probe hybridizes exclusively to the unresolved, large DNA fragments at the top of the gel, and the YsI probe only to small SalI DNA fragments at the bottom of the gel (Fig. 2B). The weak signal of the YsI probe in the upper part of the gel most probably represents hybridization to uncleaved DNA rather than residual cross-hybridization to ayl, since *vice versa*, the ayl probe does not hybridize to the cleaved YsI repeats in the lower part of the gel. Thus, under stringent conditions, the ayl and YsI-specific probes allow a clear discrimination between repeats of these two related families of repetitive DNA sequences.

Table 1. Percentages of sequence similarity between the ayl and YsI probes used in this paper and probes used by other investigators

probe	family	ayl[1] probe	YsI[2] probe	reference
YsIa4/18	ayl	**84%**	62%	Wlaschek et al. (1988)
Y23Ns	ayl	**76%**	60%	Lifschytz and Hareven (1985)
YsI10/20	YsI	68%	**85%**	Wlaschek et al. (1988)
Y20Ns	YsI	76%	**81%**	Lifschytz and Hareven (1985)
Y20Nsf1	YsI	64%	**76%**	Lifschytz (1987)

Notes:
[1] The sequence of the ayl probe was described by Vogt and Hennig (1986a), also see Fig. 1
[2] The sequence of the YsI probe can be found in Fig. 1



discrimination of (non)transcribed sequences

Fig. 1 (previous page). Sequence alignment of the ay1 and YsI probes used in this study with those used by other investigators. Sequences belonging to the ay1 family are in the upper part of the alignment, those belonging to the YsI family are in the lower part. The YsIa4/18 sequence corresponds to the reverse complement of sequence positions 1315-1891 of the 2.0 kb EcoRI DNA fragment of phage YsIa4 (Wlaschek et al. 1988). This fragment was used for transcript *in situ* hybridization by Trapitz et al. (1988). The ay1 PROBE sequence is that of the original 393 bp EcoRI ay1 DNA fragment defined by Vogt and Hennig 1986a), and for the sake of the alignment, it is represented as a tandem repeat with the first repeat consisting of sequence positions 108-393 and the second of positions 1-307. In this alignment, a single 393 bp ay1 repeat is indicated by the arrows between the AluI sites in the ay1 sequence. The Y23Ns sequence is a 240-bp sequence from a 0.67 kb EcoRI DNA fragment determined by Lifschytz and Hareven (1985) and used for *in situ* hybridization by these authors and also by Lifschytz et al. (1983). The YsI PROBE is a 562-bp SalI DNA fragment subcloned from phage DhNo255 (Chapter 2). The YsI10/20 sequence corresponds to the reverse complement of positions 1-600 of an 1142 bp SalI DNA fragment from phage YsI10 (Wlaschek et al. 1988). This fragment was used for *in situ* hybridization by Trapitz et al. (1988, 1992). The Y20Ns sequence is a partial, 240 bp-long sequence of the 720 bp SalI DNA fragment subcloned from a 1.75 kb EcoRI DNA fragment (Lifschytz and Hareven 1985; Lifschytz 1987). Y20Nsf1 is the complete sequence of the 516 bp SalI DNA fragment subcloned from the same EcoRI fragment; shown here is the reverse complement of the sequence described by Lifschytz (1987). The complete 1.75 kb EcoRI fragment was used for *in situ* hybridization by Lifschytz et al. (1983) and by Lifschytz and Hareven (1985). In all repeats, conserved restriction sites are boxed

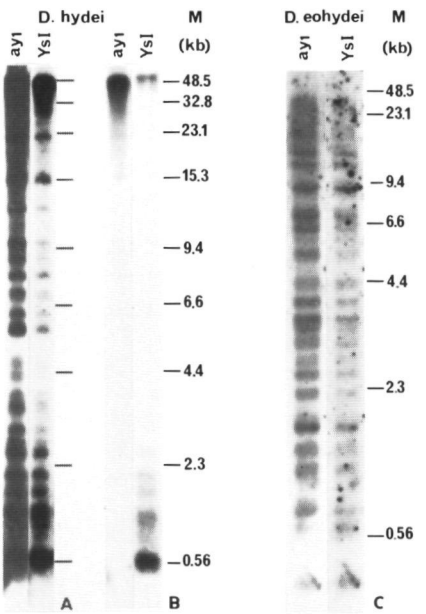


Fig. 2 A-C. Hybridization of ay1 and YsI probes to genomic DNA of *D. hydei* (**A** and **B**) and *D. eohydei* (**C**). Genomic DNA of wild-type males of *D. hydei* and *D. eohydei* was digested with SalI, separated on a 0.45% or a 0.8% agarose gel, respectively, immobilized on Hybond-N membranes and hybridized with gel-purified ay1 or YsI probes, [^{32}P]-labelled by nick translation. About 3 µg of DNA was isolated in each lane. **A** Blots were first washed in 2 x SSC/0.1% SDS at 65°C and exposed for 10 days using two intensifying screens. Under these conditions, both probes produce indistinguishable hybridization patterns. **B** Blots were then washed in 0.1 x SSC at 65°C and exposed again for 2 weeks using two screens. Under these stringent conditions, both probes hybridize exclusively to repeats of their own family. **C** Hybridization of the *D. hydei* ay1 and YsI probes to Southern blots of SalI-digested genomic DNA of *D. eohydei*. Washing conditions were 2xSSC at 65°C, exposure was 2 weeks with two intensifying screens. After washing in 0.1xSSC at 65°C, no signals were detected with either probe, even after 6-week exposures

The YsI-specific probe fails to hybridize to Nooses *transcripts under stringent conditions*

If repeats of both families are transcribed in the *Nooses* lampbrush loop pair, both ay1 and YsI probes should hybridize to loop transcripts, even under very stringent conditions. We therefore performed a series of transcript *in situ* hybridization experiments on fixed testis tissue of wild-type males. In these experiments hybridization stringency was gradually increased by raising the hybridization temperature from 65°C to 80°C (Fig. 3). If the ay1 and YsI probes are hybridized in 2 x SSC at 65°C, both probes hybridize to *Nooses* transcripts with comparable signal intensities. However, the YsI signal becomes less strong if the hybridization temperature is raised to 74°C. At a temperature of 80°C, YsI does not hybridize at all to *Nooses* transcripts, whereas ay1 clearly does.

To confirm this result, we performed additional hybridization experiments to Northern blots prepared from total RNA from testis. As observed in earlier studies (Vogt et al. 1982; Lifschytz et al. 1983; Trapitz et al. 1988), the ay1 probe reacts with testis transcripts that are heterogeneous in size. The ay1 probe did not hybridize to testis RNA from *X/ms(Y)Q1* males, which carry a deletion of the short arm of the *Y* chromosome (Fig. 4A). Such males lack the *Nooses* loop pair and also the megabase-sized clusters of uninterrupted, tandemly organized ay1 and YsI repeats. This demonstrates that any signals seen on Northern blots prepared from testis RNA of wild-type males must be derived from sequences on the short arm of the *Y* chromosome. The Northern blots also demonstrate that ay1 repeats are not transcribed in the somatic tissues of male flies (Fig. 4A), and that only one strand is transcribed (Fig. 4B). The ay1 signal did not disappear after stringent washing. In contrast, the YsI signal was much weaker and was detectable only after non-stringent washing of the blots (Fig. 4C), consistent with the expe-

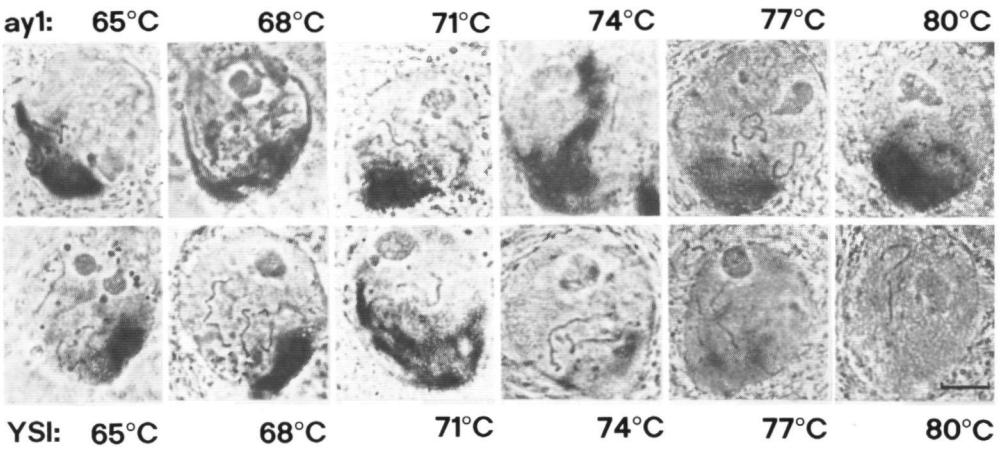


Fig. 3. Only repeats of the ay1 family are transcribed in the *Nooses* lampbrush loop pair. *In vitro* transcribed digoxigenin-11-UTP labelled "antisense" RNA probes of the ay1 and YsI families were separately hybridized to fixed primary spermatocyte nuclei of wild-type males. All hybridizations were performed in 2 x SSC at the temperatures indicated. Phase contrast. *Bar* represents 10 μm

riments of Trapitz et al. (1988). After more stringent washing this signal became completely undetectable, even after prolonged exposures.

In conclusion, the hybridization of ay1 and YsI-specific probes to *Nooses* transcripts either *in situ* or on Northern blots consistently demonstrates that repeats of the YsI family are not transcribed in this lampbrush loop pair.

YsI repeats are absent in the evolutionary more ancient species D. eohydei

D. eohydei is a species closely related to *D. hydei*. It is considered to be more ancient (Wasserman 1962; 1982). We have shown that the strand-specific co-transcription of ay1 and *gypsy* sequences in a lampbrush loop pair of *D. hydei* is conserved in this species (Chapter 5). Since in *D. hydei* the YsI repeats do not contribute to the formation of the *Nooses* lampbrush loop pair, we investigated whether *D. eohydei* contained only the more ancient ay1 family, or both ay1 and its evolutionary derivative YsI. Also in this species, ay1 probes hybridize only with

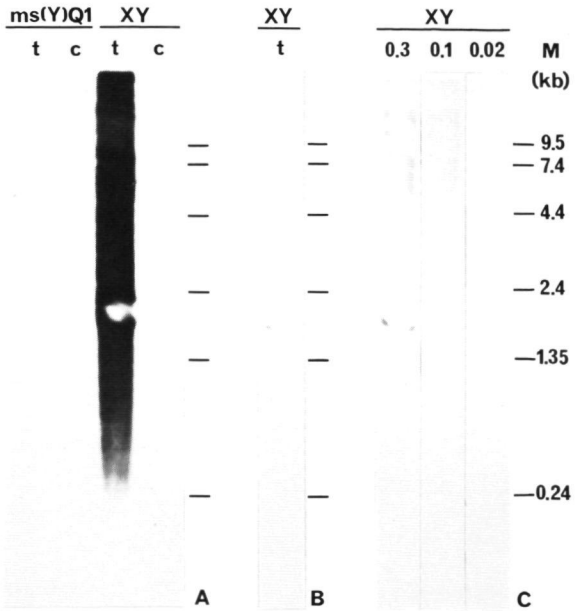


Fig. 4. A–C. Hybridization of ay1 and YsI probes to Northern blots of RNA. Total RNA was isolated from testis or from carcass of *X/ms(Y)Q1* and wild-type males, separated in a 1% denaturing gel, immobilized on Genescreen Plus membranes and hybridized with *in vitro* transcribed [^{32}P]-labelled ay1 probes or with YsI insert DNA labelled with [^{32}P] by nick translation. 20 µg RNA was loaded in each lane. Blots were successively washed in 0.3 M, 0.1 M and 0.02 M sodium-phosphate buffer, all at 50°C. **A** After washing under stringent conditions (0.02 M buffer) the "anti-sense" strand of ay1 hybridizes to testis RNA of wild-type males but not to that of males lacking the short arm of the *Y* chromosome (lanes labelled *t*). There is also no hybridization to total RNA from carcass (lanes labelled c). The transcripts that hybridize to ay1 have no specific size. The largest transcripts are in the zone of limiting mobility, the smallest are a few hundred bp long. At the level of about 2 kb, the ay1 signal is distorted by co-migrating ribosomal RNA. Exposure time was 24 hours with two intensifying screens. **B** The "sense" strand of ay1 does not react with testis RNA after washing under identical conditions. Same exposure as in A. **C** Nick translated YsI DNA only weakly hybridizes to total testis RNA after washing under non-stringent conditions (0.3 M buffer; exposure 2 weeks with two intensifying screens). The signal gradually disappears after subsequent washing under more stringent conditions (0.1 M and 0.02 M buffer, exposure times 2 weeks and 6 weeks, respectively, with two screens)

genomic DNA from males, indicating that ay1 repeats are located exclusively on the *Y* chromosome (Vogt et al. 1986).

We hybridized the ay1 and YsI probes on Southern blots containing SalI-digested genomic DNA from *D. eohydei* males. In *D. hydei*, this enzyme specifically cleaves YsI repeats but not ay1 repeats (Fig. 2A,B). Unfortunately, hybridization stringency can not be used as a criterion to distinguish between ay1 and YsI repeats of *D. eohydei*. Both probes hybridize only under non-stringent conditions, and have identical hybridization patterns in SalI digested genomic DNA of this species (Fig. 2C). Thus, all DNA sequences that hybridize to either probe are located within DNA fragments that are cleaved by SalI. This result indicates that, if both families are present in *D. eohydei*, they both have SalI sites, or alternatively, that the evolutionarily less ancient YsI family is absent in this more ancient species.

We favor the latter possibility because (i) the ay1 probe hybridizes more strongly to genomic DNA from *D. eohydei* than the YsI probe, (ii) the YsI repeat fragment size of 550-600 bp, that is typical for *D. hydei*, is not found in *D. eohydei* (Fig. 2C), and (iii) *in situ* hybridization of the YsI probe to loop transcripts in *D. eohydei* is only possible at lower temperatures than those at which the ay1 probe hybridizes (Fig. 5).

A comparison between the hybridization patterns of ay1 on Southern blots of SalI-digested genomic DNA from the two species indicates that the number of ay1 repeats in *D. eohydei* is much lower than in *D. hydei*. The same conclusion has been drawn from a comparison of EcoRI blots (Vogt et al. 1986). Large uncleaved SalI DNA fragments that are not resolved in ordinary agarose gels, are absent in *D. eohydei*, indicating that the *Y* chromosome of this species does not contain an extended cluster of tandemly organized ay1 repeats. Also, *in situ*

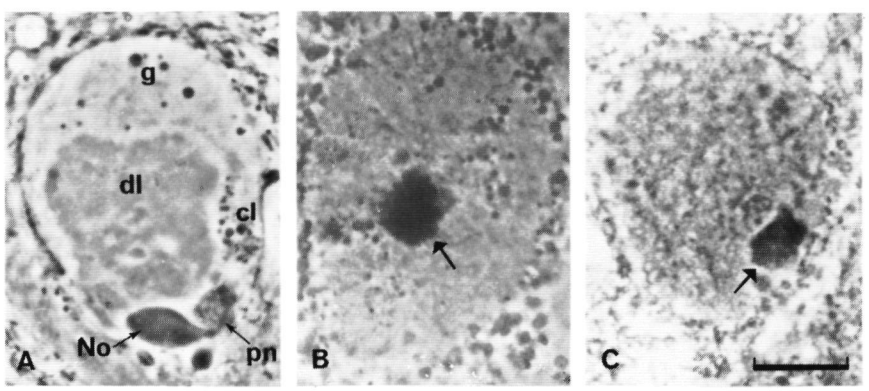


Fig. 5. A-C. Strand-specific transcription of ay1 and YsI in a *Nooses-like* lampbrush loop pair of *D. eohydei*. **A** Primary spermatocyte nucleus of *D. eohydei*, showing the loop pairs *grana* (*g*), *club-like loop* (*cl*), *pseudonucleolus-like loop* (*pn*) and *diffuse loop* (*dl*) (Hennig 1978). The *Nooses-like* loop pair (indicated by an *arrow* in **B** and **C**) is only visible after transcript *in situ* hybridization using the digoxigenin-11-UTP labelled "anti-sense" ay1 (**B**) or YsI (**C**) RNA probes. Hybridizations were in 50% formamide/6 x SSC at 50°C for ay1 and at 47°C for YsI. At higher temperatures the YsI probe did not give detectable signals. *Bar* repesents 10 μm

hybridization of ayl probes on metaphase chromosomes results in poor labelling of the short arm of the *D. eohydei Y* chromosome (Hareven et al. 1986; Vogt et al. 1986). Further, the comparison of the size of the hybridization signals in primary spermatocyte nuclei of the two species (Figs. 2 and 5) indicates that the *D. eohydei Nooses*-like lampbrush loop is considerably smaller than the *Nooses* loop of *D. hydei*.

Although we have not determined the DNA sequence of the *Y*-specific repeats that are transcribed in the *Nooses*-like loop pair of *D. eohydei*, we conclude from these findings that these repeats belong to the ayl family and not to the Ysl family. The ayl repeats of *D. eohydei* certainly differ from those in *D. hydei*, since we have failed to amplify ayl repeats from genomic DNA of *D. eohydei* by the polymerase chain reaction, using primers that correspond to the most conserved sequences of *D. hydei* ayl repeats (data not shown).

Discussion

YsI does not contribute to the lampbrush loop formed by fertility gene Q

The transcription of the *Y*-specific YsI family of repetitive DNA sequences is not beyond dispute, since several reports, even recently, claim transcription of both the ayl and the related YsI family in the *Nooses* lampbrush loop pair of *D. hydei* (Lifschytz et al. 1983; Lifschytz and Hareven 1985; Trapitz et al. 1992), whereas other reports claim only transcription of ayl (Trapitz et al. 1988). We have shown in this paper, using probes that specifically hybridize either to ayl or to YsI repeats, that members of the YsI family are not transcribed. Therefore, earlier claims that both YsI and ayl sequences are transcribed in the *Nooses* loops are based on the observation of cross-hybridization to transcribed ayl repeats of probes containing YsI sequences. As estimated from genomic clones containing ayl and transcribed *Y*-associated DNA sequences, the ayl family represents about two-thirds of the 260 kb *Nooses* transcription unit (Chapters 2, 5 and 7). Therefore, the *Nooses* transcripts provide a large target for the hybridization of other sequences that cross-hybridize with ayl.

We also have no evidence for the existence of two separate *Nooses* loop pairs, as was originally suggested by Hess (1967a). Following this idea, Lifschytz et al. (1983) concluded that ayl is transcribed in one, and YsI in the other *Nooses* loop pair. However, when both the ayl and the YsI probe were simultaneously hybridized under non-stringent conditions to transcripts *in situ*, we never observed labelling of two separate *Nooses* loop pairs (not shown).

Several other observations are in accordance with our conclusion that the YsI repeats are not transcribed. The position of the *Nooses* loop pair on the short arm of the *Y* chromosome coincides with that of the distal end of the ayl repeat cluster, but not with that of the YsI repeat cluster (Chapter 3). In more than 400 kb of genomic clones containing YsI repeats we did not detect *Y*-associated sequences, such as *gypsy* retrotransposons, which are specifically transcribed in

the *Nooses* loop pair (Chapter 2). Papenbrock (1991) isolated six different ayl-containing cDNA clones from a cDNA library constructed from total testis RNA. Although the *Y* chromosome contains about equal amounts of ayl and YsI sequences (Trapitz et al. 1992, also see Fig. 2B), not a single cDNA clone containing YsI repeats was isolated.

Fertility gene Q, *YsI. ayl. and the evolution of the* D. hydei Y *chromosome*

During the evolution of the *hydei* subgroup of the *repleta* species group, a progressive increase in the length of the *Y* chromosome occurred, but it was not accompanied by an increase in the number of the *Y* chromosomal lampbrush loop-forming fertility genes (Wasserman 1962, 1982; I. Hennig 1978; Zacharias et al. 1982). For example, the *Y* chromosome of *D. nigrohydei* is about ten times smaller than that of *D. hydei*. but it must carry all the genetic functions required for male fertility. Cytological measurements indicate that more than 90% of the *Y* chromosome of *D. hydei* does not participate in the formation of the lampbrush loops (Hess 1965b; Hennig et al. 1974b).

This latter finding is supported by our analysis of the repetitive DNA sequences on the short arm of the *Y* chromosome (Chapters 2 and 3). As estimated from metaphase chromosome preparations, the short arm contains about 6000 kb of DNA. About 260 kb are transcribed in the *Nooses* lampbrush loops (Grond et al. 1983) and about 550 kb, encoding ribosomal RNA, are transcribed in the nucleolus (Meyer and Hennig 1974). The *Y* chromosome of *D. hydei* has a length of about 43 000 kb, that of *D. eohydei* about 27 000 kb (Zacharias et al. 1982).

A part of this size difference can now be attributed to the absence of the megabase-sized clusters of ayl and YsI tandem repeats in *D. eohydei*, which in *D. hydei* together account for about one-third of the short arm (Trapitz et al. 1992; Chapter 2). The short arm of the *D. eohydei Y* chromosome carries a *Nooses*-like lampbrush loop (Vogt et al. 1986), in which both ayl and *gypsy* are transcribed with the same strand specificity as in *D. hydei* (Chapter 5). We do not know the size of this loop, but it is smaller than the *Nooses* loop of *D. hydei*. As indicated by the comparatively weak signals on Southern blots and in *in situ* hybridization experiments, the *D. eohydei Y* chromosome contains fewer ayl repeats than that of *D. hydei*. Therefore, it is not inconceivable that, unlike the situation in *D. hydei*, the majority, or all, of these repeats is located within the loop-forming transcription unit.

Thus, the megabase clusters of YsI repeats and ayl repeats are of a younger evolutionary age than fertility gene *Q*, that forms the *Nooses* loop pair. Although we cannot exclude the presence of a few YsI repeats in *D. eohydei*, the absence of YsI in this species would be consistent with the more ancient evolutionary origin of the ayl family (Wlaschek et al. 1988). By comparing the DNA sequences of ayl and YsI repeats these authors noted that the 600 bp basic YsI repeat evolved from the 400 bp basic ayl repeat by the duplication of a 200 bp sequence. In addition, we have shown that the average sequence similarity between two repeats from the YsI family is higher than between two repeats from the ayl

family (Chapter 7). It is generally assumed that the level of sequence variation among the members of a family of tandemly organized repeats increases with time (Southern 1970; Miklos 1985; Beridze 1986; John 1988).

As shown by *in situ* hybridization experiments to metaphase chromosomes, the *Y* chromosome of *D. melanogaster* carries large clusters of pentameric and heptameric satellite DNA sequences (Bonaccorsi and Lohe 1991). Most, but not all, of these clusters of repetitive sequences are at positions where loop-forming fertility genes have also been mapped (Bonaccorsi et al. 1988). For example, it has been shown that 5' AAGAC 3' repeats are transcribed in the loop pairs *A* and *C*, that are formed by fertility genes *kl-5* and *ks-1*, respectively (Bonaccorsi et al. 1990). However, there are also 5' AAGAC 3' repeats on the *Y* chromosome that do not contribute to the formation of the lampbrush loops.

Thus, it seems that, of the different members of a given family of repetitive DNA sequences on the *Y* chromosomes of *D. hydei* and *D. melanogaster*, only a subset is located within loop-forming fertility genes. It is unclear whether this distinction between transcribed and non-transcribed members of a given family is based on differences in primary DNA sequences. In the case of the *D. melanogaster* 5' AAGAC 3' repeats this is certainly very unlikely. However, further analysis of the ayl repeat family of *D. hydei*, which has a much larger sequence complexity compared to the transcribed satellite repeats of *D. melanogaster*, may reveal whether such a distinction can be made (see Chapter 7).

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CHAPTER 5

Transcription of *gypsy* elements in a *Y* chromosomal male fertility gene of *Drosophila hydei*

Submitted for publication

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Abstract. The *Y* chromosome of *Drosophila* carries genes that are essential for male fertility. During the meiotic prophase, the large transcription units of several of these genes form lampbrush loops. We have found that defective, truncated *gypsy* retrotransposons are constituents of the loop pair *Nooses* of *D. hydei*. The *Nooses* loop is a single transcription unit containing an estimated 260 kb of DNA. Throughout the loop, *gypsy* is intermingled with tandem repeat clusters of the *Y*-specific ayl family. Only one strand of ayl and only the coding strand of *gypsy* are present in loop transcripts, which have no specific size, are not polyadenylated, and do not migrate to the cytoplasm. Our data show that the loop-forming fertility genes of *D. hydei* mainly consist of *Y*-specific, satellite-like repeats that are interspersed with defective retrotransposons.

Introduction

About 40 families of transposable elements reside in the genome of *Drosophila melanogaster* (Berg and Howe 1989; Finnegan 1990). The most abundant type of transposable elements are called retrotransposons, as they have structural similarity with retroviruses. At least 19 different families of retrotransposons have been identified in this species. They are implicated in the majority of spontaneous mutations (Green 1988), and a wealth of data exists on their structure, genomic and phylogenetic distribution, and on the mechanisms by which they affect normal patterns of gene expression.

In order to cause heritable changes, retrotransposons must transpose in cells of the germ line. This requires an RNA intermediate, as has been shown for the IAP sequence of the mouse (O. Heidmann and T. Heidmann 1991), and also for several retroposons, as for example the *L1* element of the mouse (Evans and Palmiter 1991) and the *I* factor of *D. melanogaster* (Pelisson et al. 1991; Jensen and T. Heidmann 1991). Therefore, such elements must be transcribed during oogenesis or spermatogenesis. Whereas the expression of the *I* factor during oogenesis has been studied in detail (Lachaume et al. 1992; McLean et al. 1993), and *HeT-A* elements have been shown to transpose specifically in the male germ line (Biesmann et al. 1990, 1992), surprisingly little is known about the expression of retrotransposons in germ line cells of *Drosophila*, even though the promoters of several retrotransposons have been identified (see for *mdg3*: Arkhipova et al. 1986; for *copia*: Sneddon and Flavell 1990; for *mdg1*: Arkhipova and Ilyin 1991; for *gypsy*: Jarrell and Meselson 1991). For some families, the developmental pattern of expression has been determined (Parkhurst and Corces 1987), but since these studies were based on RNA extracted from entire animals, with males and females mixed, they reveal nothing about retrotransposon transcription in either the male or the female germ line.

Previous investigations of our laboratory on the molecular structure of the lampbrush loop-forming male fertility genes on the *Y* chromosome of *D. hydei* (reviewed by Hennig et al. 1989; Hennig 1990), have revealed that retrotransposons of the *micropia* family (Lankenau et al. 1988) are transcribed in the lampbrush loop pairs *Threads* and *Pseudonucleolus* (Huijser et al. 1988). The *Y* chromosome carries at least seven male fertility genes that are equally indispensible for com-

pleting the final stages of spermatogenesis (Leoncini 1977; Hackstein et al. 1982; Hackstein 1987). They are transcribed only in the male germ line and only during meiotic prophase, when five of these genes form giant lampbrush loop pairs that can be seen as prominent structures in the nuclei of primary spermatocytes.

The loops are large transcription units with estimated sizes of 260 kb for the *Nooses* (Grond et al. 1983) and up to 1500 kb for other loop pairs (Glätzer and Meyer 1981; de Loos et al. 1984), and they consist mainly of repetitive DNA sequences without obvious protein coding capacity (Vogt et al. 1982; Lifschytz et al. 1983; Huijser and Hennig 1987; Wlaschek et al. 1988; Trapitz et al. 1988). The loop transcripts accumulate large amounts of proteins that are encoded by genes on other chromosomes (Hulsebos et al. 1984; Glätzer 1984; Kremer et al. 1986; Glätzer and Kloetzel 1986; Wang et al. 1992). These properties are shared with the loop pairs formed by the *Y* chromosomal fertility genes of *D. melanogaster* (Gatti and Pimpinelli 1992).

In this paper we show that members of the *gypsy* family are abundantly transcribed in the germ line of wild-type *D. hydei* males. These *gypsy* elements are located in the lampbrush loop pair *Nooses*, that is associated with male fertility gene *Q* on the short arm of the *Y* chromosome. The *gypsy* elements are co-transcribed with repeats of the *Y*-specific ay1 family of repetitive DNA sequences, that was earlier identified as a major constituent of the *Nooses* DNA (Vogt and Hennig 1986a,b; Chapters 2 and 3).

Materials and methods

Drosophila stocks. Both the *D. hydei* Tübingen wild-type stock and the *D. eohydei* wild-type stock were from our laboratory collection. *D. hydei* males of the genotype *X/ms(Y)Q1* were used as a control, since they lack the short arm of the *Y* chromosome, and therefore, they lack fertility gene *Q*. A more detailed description of the history and the maintenance of the *ms(Y)Q1* chromosome is given in Chapter 4. Absence of the short arm was confirmed by inspection of neuroblast metaphases of *X/ms(Y)Q1* third instar larvae, and by the failure of an ay1 repeat probe to hybridize to Southern blots of genomic DNA of *X/ms(Y)Q1* adults. Repeats of the *Y*-specific ay1 family are located exclusively on the short arm of the *Y* chromosome (Vogt and Hennig 1983). The flies were grown at 18°C or 24°C as described (see Chapter 2).

Nucleic acid probes. Two probes were used for the detection of *Nooses* transcripts. As a probe for detecting transcripts of the *Y*-specific ay1 family of repetitive DNA sequences, we used an *EcoRI* fragment of 393 basepairs (bp) which, as shown by Vogt and Hennig (1986a) represents the sequence complexity of the ay1 family of repetitive DNA sequences. This fragment is called ay1. As a probe for detecting transcripts of *Y*-associated sequences of the *Nooses* loop pair, we used the 5.8 kb BamHI-EcoRI fragment of genomic clone DhNo90 (Chapter 2). Both fragments were subcloned in the pBluescript II K/S+ plasmid vector (Stratagene).

Isolation of nucleic acids. RNA was isolated from testes of 3-5 day old adult males by the method of Chirgwin et al. (1979), as described in detail by Brand and Hennig (1989). For the separation of polyadenylated and non-polyadenylated testis RNA, the mRNA purification kit of Pharmacia was used. Plasmid DNA was isolated according to a boiling procedure recommended by Stratagene.

DNA sequence analysis. Restriction fragments for DNA sequencing were subcloned in M13mp18 or M13mp19 vectors, and sequences were determined following the dideoxy chain-termination method, all as described by Amersham (1984). DNA sequences were analyzed using the software package of the University of Wisconsin Genetics Computer Group (Devereux et al. 1984). For sequence database searches and DNA sequence alignments we used the programs FASTA and LFASTA, respectively (Pearson and Lipman 1988).

Labelling of probes. Single-standed RNA probes were prepared by *in vitro* transcription, using either T3 or T7 polymerase (Stratagene), from linearized plasmid DNA, following protocols from Boehringer Mannheim. Probes for hybridization to Northern blots were labelled by incorporation of [^{32}P]-UTP. Probes for *in situ* hybridization were labelled either by incorporation of digoxigenin-11-UTP or biotin-16-UTP (both from Boehringer Mannheim). For some of the hybridizations to Northern blots, purified ay1 insert DNA was labelled by nick translation by incorporation of [^{32}P]-dCTP, following conventional protocols (Sambrook et al. 1989).

Hybridization to Northern blots. Samples of testis RNA were denatured by glyoxal/dimethylsulfoxide, separated in 1%-2% agarose gels, transferred to Gene Screen Plus membranes, hybridized, and washed as described by Brand and Hennig (1989). Approximately 20 µg total RNA, 20 µg poly-(A)$^-$ RNA, or 2 µg poly-(A)$^+$ RNA was loaded in each lane.

Transcript in situ hybridization. Transcript *in situ* hybridization to squashed testis was performed by a modification of the method of Tautz and Pfeifle (1988), as described in detail in Chapter 2. If only a single probe was used, we used digoxigenin for probe labelling. In this case, probe detection was by an anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim), and the probe was visualized by conventional phase contrast microscopy. If two probes were hybridized simultaneously, one probe was labelled with digoxigenin-11-UTP, and the other with biotin-16-UTP. In this case, probe detection was by indirect immunofluorescence, following essentially the same procedure as described in Chapter 3, except that digoxigenin was detected by successive incubations with rhodamin-conjugated sheep anti-digoxigenin Fab-fragments (Boehringer Mannheim, 1:20 dilution), Texas Red-conjugated rabbit anti-sheep antibodies (Jackson Immunoresearch Laboratories, West Grove, USA, 1:100 dilution), and Texas Red-conjugated donkey anti-rabbit antibodies (Jackson Immunoresearch, 1:100 dilution). Probe visualization by fluorescence microscopy, digital image recording, and computer-assisted image processing were as described in Chapter 3.

Results

Co-transcription of ay1 and Y*-associated sequences in the* Nooses *lampbrush loop pair*

The *gypsy* elements were identified in genomic clones that were isolated as potential segments of the lampbrush loop pair *Nooses* on the short arm of the *Y* chromosome. Our previous molecular studies revealed that the *Y*-specific ay1 family of repetitive DNA sequences accounts for about two thirds of the 260 kb of DNA transcribed in this loop pair, but that in addition, other sequences are transcribed in the loops that are also present on other chromosomes, so-called *Y*-associated DNA sequences (Vogt and Hennig 1983, 1986a,b; also see Chapter 2).

Using ay1 repeats as a probe to screen genomic libraries, we recovered 300 kb of genomic DNA in plasmid, lambda and cosmid clones containing both ay1 and *Y*-associated DNA sequences (see Chapter 2).

Three of the lambda clones are shown in Fig. 1. Each clone contains ay1 repeats, that are organized in one to several clusters of tandem repeats. In addition, they also share *Y*-associated sequences. In clone DhNo86, the shared sequence is located in a 3.8 kb BamHI-HindIII fragment, in clone DhNo90 in a 5.8 kb BamHI-EcoRI fragment, and in DhNo19 in a 3.7 kb EcoRI-EcoRI fragment. These fragments are designated DhNo86BH3.8, DhNo90BE5.8 and DhNo19EE3.7, respectively. On Southern blots containing purified lambda DNA, the fragments cross-hybridize with one another under non-stringent conditions. Under stringent

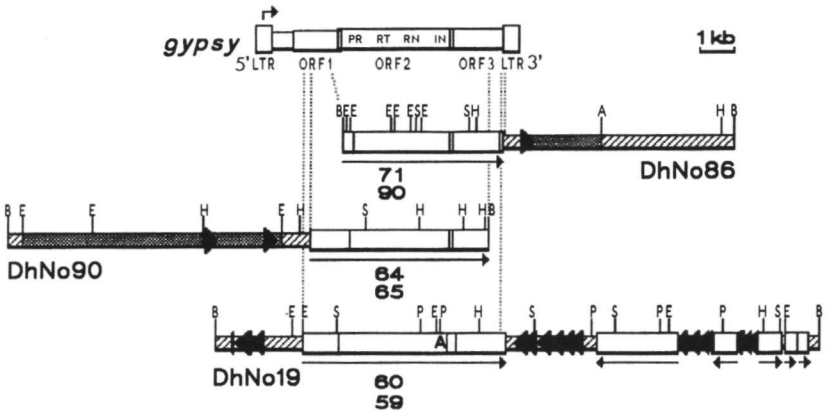

Fig. 1. Alignment of *D. hydei Y*-associated *gypsy* sequences with the *gypsy* element of *D. melanogaster*. Restriction maps of three ay1-containing lambda clones are shown. *Gypsy* sequences are indicated as open rectangles, restriction fragments hybridizing to ay1 probes are indicated by dark shading. Individual ay1 repeats, as identified by sequence comparison with the basic 393 bp ay1 repeat defined by Vogt and Hennig (1986a), are indicated by black arrowheads, which also indicate the direction of transcription of ay1 in the *Nooses* loop pair. Restriction fragments hybridizing neither to ay1 nor *gypsy* probes are hatched. The sequenced parts of these fragments have no obvious similarities to any sequence in the EMBL database (Release 35, June 1993). For each *gypsy* sequence the direction of transcription of the coding strand is indicated by an arrow. The numbers below the *gypsy* fragments indicate the percentage of sequence similarity to the corresponding sequences from the *gypsy* elements of *D. melanogaster* (upper numbers) and *D. virilis* (lower numbers). In the *D. melanogaster gypsy* element at the top, the long terminal repeats (LTR), open reading frames (ORF) as well as the positions of the protease (PR), reverse transcriptase (RT), ribonuclease (RN) and integrase (IN) activities encoded by ORF2, are indicated. The start site of *gypsy* transcription is indicated by the small arrow above the 5' LTR. In the largest *gypsy* sequence of DhNo19, the A indicates a poly(A)-tail which is located between ORF2 and ORF3. Restriction enzyme abbreviations are A, AvaI; B, BamHI; E, EcoRI; H, HindIII; P, PstI and S, SalI. The complete nucleotide sequence of DhNo19 has been submitted to the EMBL database under accession number X74538, the partial sequence of DhNo86 has been submitted under accession numbers X74539, X74540, X74541 and X74542, and the partial sequence of DhNo90 under accession numbers X74536, X74537 and X74543

conditions, they all hybridize to different positions on other chromosomes, with most copies in the centromere-associated heterochromatin of the *X* chromosome and the autosomes, and most importantly, they also hybridize to *Nooses* transcripts *in situ* (Chapters 2 and 3). Therefore, the three clones shown in Fig. 1 represent potential fragments of the *Nooses* lampbrush loop pair.

If this assumption is correct, this *Y*-associated sequence must give the same hybridization pattern to *Nooses* transcripts as ay1. We tested this by fluorescent transcript *in situ* hybridization, using a biotin-labelled, single-stranded RNA probe corresponding to ay1, and a digoxigenin-labelled RNA probe corresponding to DhNo90BE5.8. This fragment was chosen because it is present in at least four different ay1-containing genomic clones (Chapter 2), and it may, therefore, occur

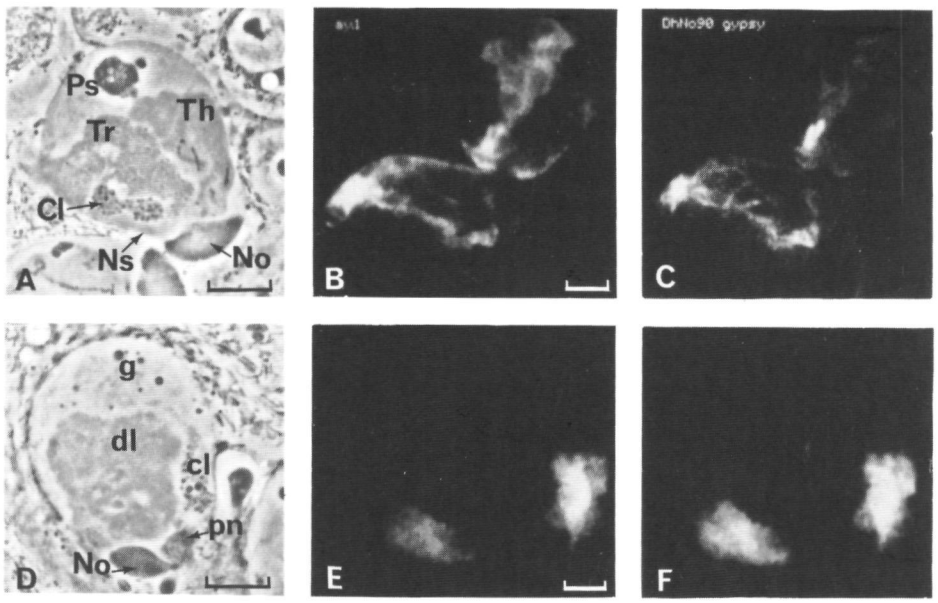

Fig. 2 A–F. Co-transcription of ay1 and *gypsy* in the *Nooses* loop pair of *D. hydei* and in a loop pair of *D. eohydei*. In **A** and **D**, a primary spermatocyte nucleus is shown for each species (phase contrast). The loop pairs of *D. hydei* (**A**) are Th, *Threads*; Ps, *Pseudonucleolus*; Cl, *Clubs*; Tr, *Tubular ribbons*; Ns, *Nooses* (Hess and Meyer 1968). Those of *D. eohydei* (**D**) are g, *granular loop*; cl, *club-like loop*; dl, *diffuse loop* and pn, *proximal loop* (I. Hennig 1978). Nucleolus organizers are marked *No*. Fixed testis tissue of *D. hydei* (**B** and **C**) and *D. eohydei* (**E** and **F**) was hybridized simultaneously with a probe for the original ay1 repeat and a probe for the DhNo90BE5.8 fragment, which contains a *gypsy* sequence. Single-stranded RNA probes were prepared by *in vitro* transcription of linearized pBluescript II KS+ plasmids, using biotin-16-UTP to label the ay1 probe, and digoxigenin-11-UTP to label the *gypsy* probe. The ay1 probe was detected by fluorescein isothiocyanate fluorescence (**B** and **E**), the *gypsy* probe by Texas Red fluorescence (**C** and **F**). Two nuclei, each containing one labelled loop pair, are shown for each species. In *D. hydei* both probes give an almost identical signal on the *Nooses* loop pair. In *D. eohydei*, both probes label a loop pair that does not correspond to any of the four pairs described earlier. Hence, *D. eohydei* also has five lampbrush loop pairs on the *Y* chromosome, the same number as *D. hydei*. In all figures, the *bar* indicates 10 μm

multiple times in the transcribed DNA of the *Nooses* loop pair. As shown in Fig. 2, the two signals completely overlap and cover the entire *Nooses* loop pair. Also in *D. eohydei*, a species closely related to *D. hydei* (Wasserman 1982) both sequences are co-transcribed in a lampbrush loop pair formed by a *Y* chromosomal fertility gene.

This result indicates that throughout the *Nooses* loop pair, both types of DNA sequences are intermingled, and that no major parts of the transcription unit are devoid of either sequence. It also confirms our earlier metaphase *in situ* hybridization experiments, which revealed that all *Y* chromosomal copies of DhNo90BE5.8 are clustered distally on the short arm of the *Y* chromosome, at a position where also ay1 repeats are located (Chapter 3). From genomic Southern blots we estimated that this region of the *Y* chromosome contains about ten copies of DhNo90BE5.8, ten copies of the related sequence in DhNo86BH3.8, and at least two copies of the related sequence in DhNo19EE3.7 (Chapter 2). Since these blots did not provide evidence for a tandem repetition of any of these sequences, they must all be intermingled with other sequences, most likely with ay1 repeats.

Also hybridization experiments to Northern blots prepared from total testis RNA of *D. hydei* imply that both sequences occur in *Nooses* transcripts, since both ay1 and DhNo90BE5.8 hybridize only to testis RNA if the short arm of the *Y* chromosome, carrying fertility gene *Q*, is present (Fig. 3). In testis RNA from (sterile) males which lack the short arm of the *Y* chromosome, and therefore lack fertility gene *Q* and the associated loop pair *Nooses*, no hybridization is seen with either probe. Further, both probes hybridize to RNA fragments of a heterogeneous size. The largest fragments are 10 to 20 kb, and the smallest are only a

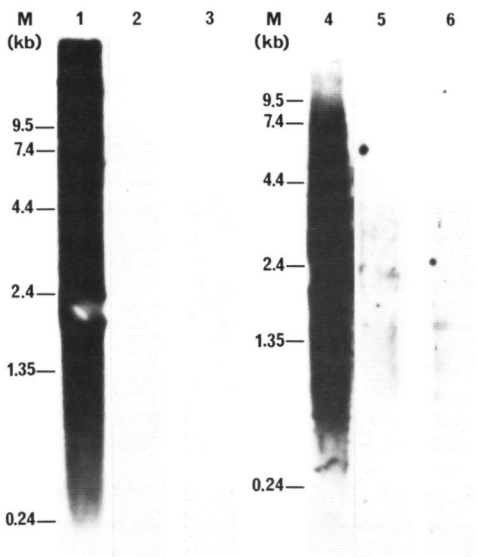

Fig. 3. Testis transcripts containing ay1 and *gypsy* sequences have a heterogeneous size and are detected only in males carrying the *Nooses* loop pair. 20 µg total testis RNA of wild-type *D. hydei* males (lanes 1, 3, 4, and 6) or males of the genotype *X/ms(Y)Q1* (lanes 2 and 5) was loaded in each lane. Blots were hybridized with [^{32}P]-labelled strand specific probes for ay1 (lanes 1 to 3) or for DhNo90BE5.8 (lanes 4 to 6). All membranes were stringently washed in 0.02 M sodium phosphate buffer at 50°C, and exposed for 48 h using two intensifying screens. The ay1 probe hybridizes to testis transcripts of a heterogeneous size, but only if the short arm is present (lanes 1, 2), and so does the probe for the coding strand of *gypsy* (lanes 4, 5). Under identical conditions, probes for the opposite strand of ay1 (lane 3) and the non-coding strand of *gypsy* (lane 6) do not hybridize. At a level corresponding to approximately 2kb, co-migrating ribosomal RNA causes distortions of the signals

few hundred bp. This size heterogeneity is not unexpected given the fact that the growing, nascent loop transcripts display a clear size gradient (Grond et al. 1983). In addition, current methods of RNA isolation are not suited to allow transcripts of sizes of several hundred kb to be isolated without degradation.

The Northern blots also reveal that only one strand of DhNo90BE5.8 is transcribed, consistent with earlier *in situ* hybridization experiments (Chapter 2). Thus, within the *Nooses* transcription unit not only all ayl repeats (Lifschytz and Hareven 1985; Trapitz et al. 1988; also see Chapter 4) but also all copies of the *Y*-associated DhNo90BE5.8 sequence have the same orientation.

Identification of defective gypsy *elements as Y-associated sequences of the* Nooses *loop pair*

We sequenced DhNo90BE5.8 and the related sequences from DhNo19 and DhNo86. As shown in Fig. 1, each of the three lambda clones contains a 4 to 5 kb long DNA sequence with a high degree of similarity to the *gypsy* retrotransposon, known from *D. melanogaster* (Marlor et al. 1986) and *D. virilis* (Mizrokhi and Mazo 1991). All these *Y*-associated *gypsy* elements of *D. hydei* are defective. They have lost their protein coding capacity, since all open reading frames are randomly destroyed by deletions or frame shifts (a more detailed description of these and other *gypsy* sequences is given in Chapter 7). In addition, the DNA sequences which in full-length *gypsy* elements control transcription seem to be absent due to truncations at either the 5' end, the 3' end, or at both ends. For example, the 5' long terminal repeat, which contains the *gypsy* promoter (Arkhipova et al. 1986; Jarrell and Meselson 1991), is absent in the *gypsy* element of DhNo90 and in the large *gypsy* element of DhNo19. Also the binding sites for the protein encoded by the *suppressor-of-Hairy wing* (*su(Hw)*) gene (Spana et al. 1988) are lacking (also see Chapter 7 for more details).

The sequence analysis of the *gypsy* fragments in DhNo86 and DhNo90 revealed that the coding strand of *gypsy* is present in the *Nooses* transcripts. To confirm this result, we determined the orientation of the ayl repeats immediately flanking the *gypsy* elements in these clones by partial sequencing of ayl repeat clusters. DhNo19 was completely sequenced as its restriction map had indicated the presence of at least three separate clusters of ayl repeats (Chapter 2). Comparisons of the orientations of adjacent *gypsy* and ayl sequences show that the *gypsy* fragments in DhNo90 and DhNo86 are indeed transcribed from the same strand as ayl (Fig. 1), suggesting that these clones represent genuine segments of the *Nooses* transcription unit.

In contrast, DhNo19 contains six different *gypsy* fragments, with only two in the same orientation as the ayl repeats, which all have the same orientation in the clone (Fig. 1). Since the antisense strand of *gypsy* is not found in *Nooses* transcripts, neither by *in situ* hybridization (Chapter 2), nor by hybridization to Northern blots (Fig. 3), the genomic clone DhNo19 cannot represent a part of the *Nooses* transcription unit. Thus, not all ayl repeats that are interspersed with *Y*-associated sequences are located within the loop. This explains our earlier finding that the

Y chromosome contains more DNA with interspersed ay1 repeats than predicted by the 260 kb length estimate for the *Nooses* transcription unit. However, clones such as DhNo19 seem to be rather exceptional, since from nine lambda and three cosmid clones in which both *gypsy* and ay1 have been identified, it is the only one with *gypsy* sequences in the opposite transcriptional orientation relative to ay1 (see Chapter 7). Thus, whereas *gypsy* can occur in both polarities with respect to ay1 repeats, only the coding strand of *gypsy* is found in *Nooses* transcripts.

Distribution of Nooses *transcripts during male germ cell development*

Because retrotransposon transcripts encode for proteins, we investigated whether the *Nooses* transcripts, containing *gypsy* sequences as well as ay1 repeats, are transported from the nucleus to the cytoplasm. To this end, we used the ay1 probe to follow the distribution of the loop transcripts during spermatogenesis in wild-type males of *D. hydei.* Identical results were obtained using the DhNo90BE5.8 *gypsy* probe (not shown). Detailed descriptions of the different stages of male germ cell development in *D. hydei* have been given by Hess and Meyer (1968), Grond (1984), Hennig (1985) and Hennig and Kremer (1990).

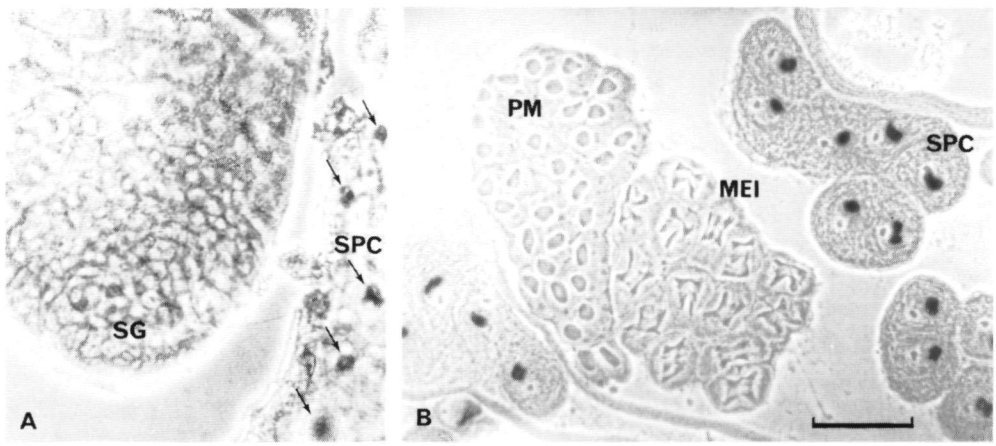

Fig. 4 A,B. Distribution of *Nooses* transcripts during spermatogenesis, as followed by transcript *in situ* hybridization using the ay1 probe. A single-stranded RNA probe was prepared by *in vitro* transcription from a linearized plasmid, using digoxigenin-11-UTP. The probe is detected using an anti-digoxigenin antibody conjugated with alkaline phosphatase. **A** In the tip of the testis tube, spermatogonia (*SG*) are not labelled, while adjacent primary spermatocytes (*SPC*) contain a labelled *Nooses* loop pair in their nuclei (indicated by arrows). This is more clearly seen in **B**. Label is found only in the nuclei of primary spermatocytes (*SPC*), there are no transcripts containing ay1 in the cytoplasm of these cells. In the center of the figure, a cyst of secondary spermatocytes (containing almost the complete number of 16 cells) during anaphase II of meiosis (*MEI*) is seen, and at the left, there is a complete cyst of 32 spermatids of an early postmeiotic stage (*PM*), with round or slightly oval Nebenkern derivatives. All cells of both cysts are completely free of label, as are all subsequent stages of spermatid differentiation. Phase contrast. *Bar* represents 100 μm

Spermatogenesis starts in the tip of the testis tube where primordial germ cells differentiate into spermatogonia, which proliferate by mitotic divisions. In such cells the *Y* chromosome is not active (Hennig 1967, 1985), and, as expected, we did not find transcripts containing ay1 or *gypsy* in such cells (Fig. 4 A).

Spermatogonia develop into primary spermatocytes, *i.e.* they enter meiotic prophase. Because primary spermatocyte development includes more than half of the

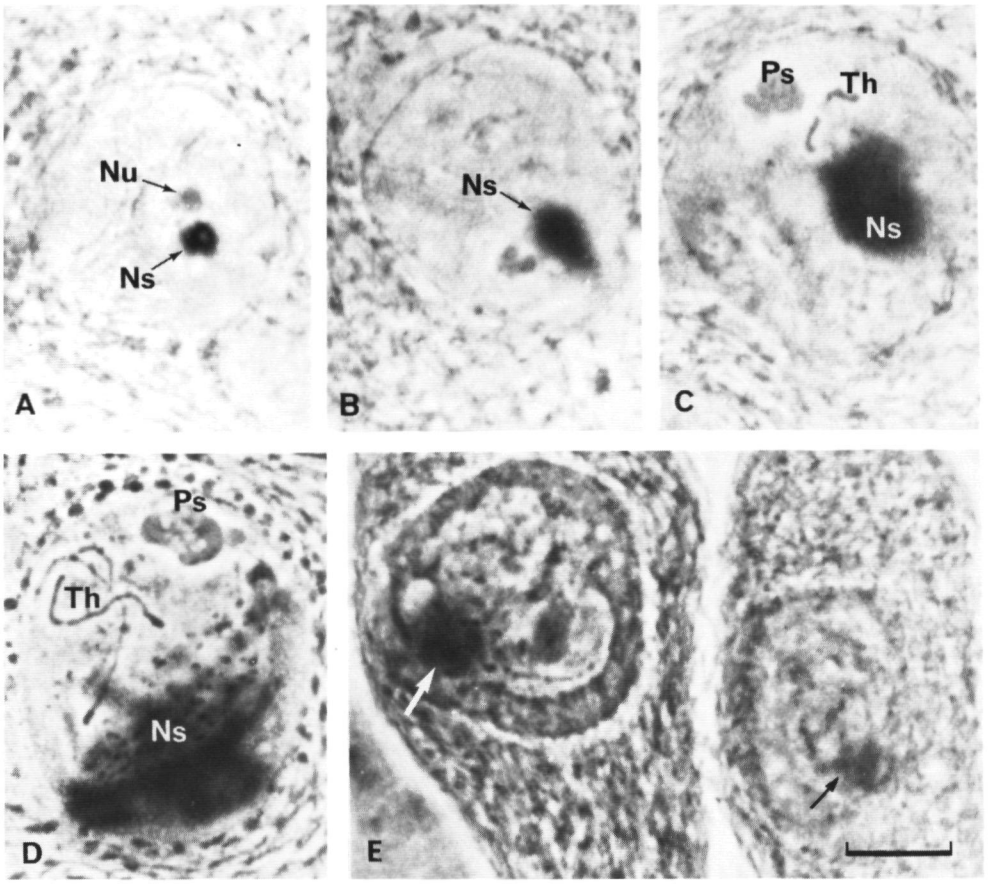

Fig. 5 A–E. Gradual unfolding of the *Nooses* loop pair during the successive stages of primary spermatocyte development. The *Nooses* loop pair was visualized by transcript *in situ* hybridization using the digoxigenin-labelled ay1 probe. **A** In Stage I primary spermatocytes, the *Nooses* loop pair (*Ns*) starts to unfold from a position close to the round nucleolus (*Nu*). The other loop pairs cannot be seen in this nucleus. **B** and **C** Subsequent, gradual unfolding of the *Nooses* loop pair, together with the other loop pairs, during Stage I. Other loop pairs seen are the *Threads* (*Th*) and the *Pseudonucleolus* (*Ps*). **D** During Stages II and III, the *Nooses*, as well as the other loop pairs, are seen at their maximum expansion. **E** During Stage IV, the nuclei become round, and the loops are degraded. In the nucleus on the left, some residual ay1-containing transcripts are present (indicated by the white arrow). In the nucleus on the right, which is about to enter metaphase I, such transcripts (indicated by the black arrow) are barely detectable. Phase contrast. *Bar* represents 10 μm

total time needed for spermatogenesis (Grond 1984; Hennig and Kremer 1990), we studied this phase in more detail (Fig. 5). The different stages of primary spermatocyte development have been defined by Hennig (1967) on the basis of their typical cytology. During Stage I, which lasts approximately 24 hours, the loop pairs start to unfold, and in nuclei of such cells, the *Nooses* can be seen as a small, round loop pair, in close proximity to the round nucleolus (Fig. 5A). Subsequently, the *Nooses* unfold, together with the other loop pairs, from a position close to the nucleolus (Figure 5B, C).

During Stage II, lasting approximately 90 hours, all loops have reached their maximum size, and the primary spermatocytes begin a period of growth and intense transcriptional activity (Fig. 5D). The nucleolus is large and is attached to the nuclear membrane. Transcriptional activity decreases somewhat down during Stage III (27 hours), even though the loops remain fully expanded (Hennig 1967).

During the short Stage IV (4 hours), the nuclei become round, the nucleolus detaches from the nuclear membrane, and RNA synthesis ceases (Hennig 1967). In primary spermatocytes of this stage, the signal of the ayl probe is reduced in intensity and residual *Nooses* transcripts can be seen between the fragments of the other loop pairs (Fig. 5E, left nucleus). Thus, together with the transcripts of the four other loop pairs, the *Nooses* transcripts are rapidly decomposed at the end of meiotic prophase. While some, probably proteinaceous, remnants of the *Pseudonucleolus* and the *Clubs* may still be detectable during the first meiotic division (Hess and Meyer 1968), almost no ayl-containing transcripts have remained at the end of primary spermatocyte Stage IV (Fig. 5 E, right nucleus). As a consequence, such transcripts are not found during the second meiotic division and the subsequent postmeiotic stages (Fig. 4 B).

Because sense transcripts of retrotransposons are polyadenylated (Berg and Howe 1989), we also investigated whether the giant *Nooses* lampbrush loop transcripts have poly(A) tails. As documented in Fig. 6, transcripts containing ayl repeats are detectable only in poly(A)$^-$ testis RNA, but not in poly(A)$^+$ testis RNA.

To summarize, the *Nooses* transcripts containing ayl or *gypsy* sequences are of a heterogeneous size, do not migrate to the cytoplasm, and are not polyadenylated. These transcripts are detectable only within the nuclei of primary spermatocytes, in close association with the DNA axis of the lampbrush loop pair, and they are degraded, together with the transcripts of the other loop pairs, shortly before the first meiotic division. These observations are consistent with those of Bonaccorsi et al. (1990) who used probes containing 5' AAGAC 3' repeats to follow the transcripts of the loop pairs formed by fertility genes *kl-5* and *kl-3* on the *Y* chromosome of *D. melanogaster*.

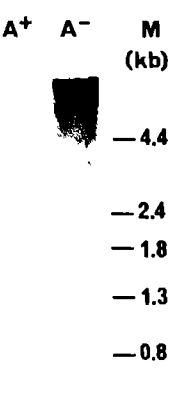

Fig. 6. Transcripts containing ayl are not polyadenylated. For this blot, 2 μg poly(A)$^+$ and 20 μg poly(A)$^-$ testis RNA were used. The blot was hybridized with DNA of the original ayl repeat that was labelled with [^{32}P] by nick translation, washed in 0.3 M sodium phosphate buffer at 65°C, and exposed for 3 days using two intensifying screens

Discussion

We have identified defective, truncated members of the *gypsy* retrotransposon family as constituents of the DNA that is transcribed in the *Nooses* lampbrush loop pair, formed by male fertility gene *Q* on the *Y* chromosome of *D. hydei*. In the loop-forming transcription unit, the *gypsy* elements occur intermingled between members of the ayl family of *Y*-specific repetitive DNA sequences that are transcribed in the *Nooses* as well. Only the sense strand of *gypsy* is present in the loop transcripts.

Probes containing *gypsy* sequences result in strong signals on *Nooses* transcripts, both *in situ* (Fig. 2, also see Chapter 2), and on Northern blots (Fig. 3), suggesting that *gypsy* represents a major constituent of the loop. We have no means to accurately assess the copy number of the *gypsy* elements in the *Nooses* transcription unit, which contains an estimated 260 kb of DNA (Grond et al. 1983). All *Y* chromosomal *gypsy* elements are clustered together at a distal position on the short arm, thereby defining the cytological position of fertility gene *Q* (Chapter 3). If all the *Y* chromosomal copies of *gypsy*, as recognized by their hybridization to either DhNo90BE5.8 or DhNo86BH3.8 (see Chapter 2), are located within the transcription unit, *gypsy* would represent at least half of the estimated 80-90 kb of *Y*-associated DNA of the loop, consistent with the strong signals on *Nooses* transcripts. However, some of the *Y* chromosomal *gypsy* elements, as for example those in clone DhNo19, are not transcribed in the *Nooses*.

Gene *Q* is not the only loop-forming male fertility gene of *D. hydei* containing defective retrotransposons. Members of the *micropia* family that have also lost their protein coding capacity are transcribed in the loop pairs *Threads* and *Pseudonucleolus*, associated with male fertility genes *A* and *C*, respectively (Huijser et al. 1988), Also in this case only the sense strand of the retrotransposon is present in the loop transcripts (D.-H. Lankenau 1993; S. Lankenau et al. 1994). Thus, each loop-forming fertility gene may contain a few, or even only one family of retrotransposons, with all members having the same orientation in the loop-forming transcription unit.

The first question raised from these observations is why these retrotransposons do not interfere with the function of the respective fertility gene. Insertions of retrotransposons into genes usually result in (spontaneous) mutations in *D. melanogaster* (Green 1988). In the case of gene *Q* we have shown that at least the *gypsy* element in clone DhNo90, which may occur mutiple times in the loop, has lost the binding sites for the *su(Hw)* protein. This protein is a zinc-finger protein (Parkhurst et al. 1988; Spana et al. 1988). In *gypsy*-induced mutations, the binding of the *su(Hw)* protein is sufficient for mediating the mutagenic effects of *gypsy* on the expression of adjacent protein-coding genes (Geyer et al. 1986; Peifer and Bender 1988; Mazo et al. 1989; Geyer and Corces 1992; Smith and Corces 1992; Roseman et al. 1993). Since a probe containing the *su(Hw)* binding sites of the *gypsy* element of *D. melanogaster* does not hybridize to *Y*-specific DNA fragments in *D. hydei*, nor to *Nooses* transcripts (Chapter 7), it would seem that all the *gypsy* elements in the loop have lost the capacity to bind the *su(Hw)* protein, explaining why these elements do not interfere with gene function.

In Miller spreads the *Nooses* loop can be seen as a single transcription unit (Grond et al. 1983). Thus, the *gypsy* elements in this transcription unit neither serve as secondary initiation sites for loop transcription, nor do they impede the normal progression of the RNA polymerase along the loop DNA, suggesting that the promoter sequences in the 5' *gypsy* LTR, and the transcriptional termination signals in the 3' LTR (Arkhipova et al. 1986; Jarrell and Meselson 1991) are either deleted, mutated, or nonfunctional in the context of lampbrush loop transcription. Consistent with the first possibility, we have found that the *gypsy* element in DhNo90 has a deletion of the 5' LTR, and that the element in DhNo86 has almost completely lost its 3' LTR.

What is the functional significance of the *gypsy* elements as constituents of fertility gene *Q*? Mutations or deletions of this gene cause a developmental arrest of spermatogenesis at the end of the elongation stage, before spermatid individualization (Hackstein et al. 1982). Since the molecular basis of this effect is unknown, it is difficult to assess the role of the transcribed *gypsy* sequences for the function of gene *Q*. However, mutant alleles of loop-forming fertility genes that do not form a loop are sterile (Leoncini 1977; Hackstein et al. 1982, 1991). Together with the high mutation frequencies of the loop-forming genes (Chapter 1) this implies that the fertility gene is an integral part of the DNA that is transcribed in the loop. Therefore, the transcription of the repetitive loop constituents, such as ay1 and *gypsy*, is required for gene function.

We have previously proposed that retrotransposons may represent an essential feature of fertility gene structure, as they contain DNA sequences important for the initiation and enhancement of transcription (Hennig 1990). The frequent inclusion of such elements in the large loop-forming transcription units, as shown here for the *gypsy* elements of the *Nooses* loop pair, might be instrumental for maintening an open chromatin conformation, necessary for the synthesis of the long loop transcripts. However, as discussed before, the *gypsy* elements in the *Nooses* specifically lack promoters and *su(Hw)* binding sites. Since the *su(Hw)* protein functions as a specific transcriptional activator of *gypsy* transcription (Parkhurst and Corces 1986; Mazo et al. 1989), it is less likely that *gypsy* is required for promoting transcription in the *Nooses* lampbrush loops.

As discussed in Chapter 1, the loop-forming fertility genes may function by protein binding or by protein coding. In the context of the first possibility, Hennig (1987) has suggested that retrotransposons insertions may function to augment the number of protein binding sites in the lampbrush loops. With respect to the second possibility, there is no direct evidence for a protein coding function of gene *Q*. We have shown in this Chapter that the large loop transcripts, as detected by ay1 or *gypsy* probes, have no specific size, are not polyadenylated, are not transported to the cytoplasm, and are absent postmeiotically, when most proteins of the sperm are being made (Hennig 1967). These findings seem to be incompatible with a protein-coding role of loop transcripts containing ay1 and *gypsy*. In addition, sequence analysis of *Y*-associated *gypsy* elements (Chapter 7) (and also that of ay1 repeats, see Chapter 6), indicates that the point mutations and deletions accumulated by these sequences are unlikely to interfere with the function of gene *Q*. Moreover, ay1 and *gypsy* are absent in the lampbrush loops

of most *Drosophila* species (see Chapters 1 and 7). Therefore, it seems that the function of the lampbrush loop-forming male fertility genes does not depend on the particular type of repetitive DNA sequences that occupy the bulk of the loops (also see Hennig 1990 for discussion).

However, with the current state of knowledge, it is impossible to exclude a protein-coding function of fertility gene *Q*. This possibility has been raised by Hardy et al. (1981), Goldstein et al. (1982), and, most recently, by Gepner and Hays (1993) for at least one of the loop-forming genes on the *Y* chromosome of *D. melanogaster*. The proposed protein coding function is supported by the existence of temperature-sensitive sterile alleles of several of the loop-forming genes of *D. melanogaster* (Ayles et al. 1973) and of *D hydei* (Leoncini 1977), one of which has been assigned to gene *Q* (Hackstein et al. 1982). This issue is discussed in more detail in Chapter 9.

We wish to express our gratitude to Dr Johannes Hackstein for providing us with the *T(X;Y)59/ms(Y)Q1* flies, and to Wiell Janssen for excellent technical support. We thank Dr Tom Hays for communicating unpublished data, and Prof Dr. Erwin Schmidt, Dr. Peter Vogt and our colleagues Anna Akhmanova, Koos Miedema and Xiaoping Sun for critical comments on earlier versions of the manuscript

CHAPTER 6

Sequence heterogeneity within a family of repetitive DNA sequences from a male fertility gene on the *Drosophila Y* chromosome

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Abstract. We have investigated the sequence conservation between repeats of the *Y*-specific ayl family of repetitive DNA sequences. This family, with a sequence complexity of 400 base pairs, occupies approximately two thirds of the DNA that is transcribed in the lampbrush loop pair *Nooses* on the *Y* chromosome of *Drosophila hydei*. The *Nooses* loop pair is formed by male fertility gene *Q* during meiotic prophase, and its transcription is essential for male fertility. Because the *Y* chromosome also contains ayl repeats outside of the *Nooses* transcription unit, sequences of transcribed and nontranscribed members of this family were compared. We found that transcribed repeats are not more conserved in their sequence than nontranscribed repeats. All members of the ayl family are subject to random base pair substitutions and deletions, suggesting that such changes do not interfere with fertility gene function.

Introduction

The *Y* chromosome of *Drosophila hydei* harbors at least seven genes that can be mutated to male sterility (reviewed by Hackstein 1987). Five of these genes form visible lampbrush loop pairs during meiotic prophase (Hess and Meyer 1968; Hennig 1985, 1987). We have investigated the molecular organization of the repetitive DNA sequences in the gene *Q* on the short arm of the *Y* chromosome (Vogt et al. 1982; Vogt and Hennig 1983; 1986a,b; Chapters 2, 3, 4 and 5). This gene forms the loop pair *Nooses* (Hackstein et al. 1982).

The molecular composition of the DNA that is transcribed in the *Nooses* is fairly well understood (reviewed by Hennig 1990). The loop DNA consists for about two-thirds of repeats of the *Y*-specific ayl family of repetitive DNA sequences (Chapter 2). The sequence complexity of this family is represented by a 393 bp EcoRI DNA fragment. Within the transcription unit, the ayl repeats are organized in clusters of 3-10 tandem repeats. Interspersed between the clusters, unrelated repetitive DNA sequence families were identified that have copies on other chromosomes as well (Vogt and Hennig 1986b; Chapter 2). They are called *Y*-associated DNA sequences. Several of the transcribed *Y*-associated DNA sequences have been identified as defective *gypsy* retrotransposons (see Chapters 5 and 7).

The initial sequence analysis of ayl repeats by Vogt and Hennig (1986a,b), Lifschytz and Hareven (1985) and Wlaschek et al. (1988) did not reveal an obvious function for this major DNA constituent of the *Nooses* loop pair. The sequenced repeats were heterogeneous in size due to internal deletions and duplications, and showed 80-85% sequence similarity. Several regions of ayl were found to be similar, but not identical, to the consensus core sequence of the autonomously replicating sequences (ARS) of budding yeast, or to functional sequence motifs of eukaryotic enhancers. Based on these findings, it was proposed that ayl repeats serve to create an "open" chromatin structure throughout the *Nooses* transcription unit (Vogt and Hennig 1986a,b; Hennig et al. 1989).

However, there was no direct evidence that the particular ayl repeats studied were indeed transcribed in the *Nooses* loop pair. As shown independently by Trapitz et al. (1992) and during the course of the present work (see Chapters 2 and 3),

there is a megabase-sized cluster of tandemly organized ay1 repeats proximal to the loop-forming region on the short arm of the *Y* chromosome. Thus, the *Y* chromosome contains much more DNA harboring ay1 repeats than the 260 kb of DNA transcribed in the *Nooses*. In addition, proximal to the large ay1 cluster there is a megabase-sized cluster of tandemly organized repeats of the Ys1 family. This family of *Y*-specific repetitive DNA sequences is an evolutionary derivative of ay1 (Wlaschek et al. 1988), but the members of the Ys1 family are not transcribed (Chapter 4).

In this paper, we reinvestigate the question of sequence conservation within the ay1 family by studying a larger sample of repeat sequences, including repeats from the loop as well as other, nontranscribed repeats. Based on the analysis of 76 sequenced ay1 repeats, which together contain almost 20 kb of DNA, we find that all repeats are different due to base pair changes and deletions, which both occur at any position within the repeat. The average sequence similarity between transcribed repeats is not significantly different from that between nontranscribed ay1 repeats. Thus, the major constituent of the *Nooses* loop pair lacks a conserved DNA sequence. These findings are discussed in relation to the evolution and the possible functions of the lampbrush loop-forming male fertility genes.

Materials and methods

The ay1 repeat sequence data set. For the present analysis, we collected a total of 76 different ay1 repeat sequences. Some of the sequences were taken from the literature, while the majority were sequenced by us. They were grouped into the following four classes.

Class I. Repeats derived from cDNA libraries. They represent DNA segments transcribed in the *Nooses* lampbrush loop pair (Papenbrock 1991; also see Fig. 2). This class includes 18 repeats. The sequences of 9 repeats were determined from cDNA clones, the sequences of 9 other repeats from the genomic lambda clone YslaG9S from which several of the transcribed repeats were found to originate. Therefore, the repeats not isolated as a cDNA are likely to be transcribed as well.

Class II. Repeats from genomic clones that contain ay1 and *gypsy* in an orientation consistent with the presence of the clone in the transcription unit. As discussed in Chapters 2,5 and 6, it is very likely that they are transcribed in the *Nooses* loop pair. These clones are plasmid clone MY3 (Vogt and Hennig 1986b), lambda clones DhNo86 and DhNo90, and cosmid clone DhNocos6 (see Fig. 1). This class consists of 19 different ay1 repeat sequences (see Table 1 and Fig. 3).

Class III. Repeats from genomic clone DhNo19 which contains *gypsy* sequences in both orientations relative to ay1 (see Fig. 1). Since only the coding strand of *gypsy* is detected in *Nooses* transcripts (Chapter 4), such ay1 repeats are not transcribed. This class contains 16 ay1 repeat sequences (see Table 2 and Fig. 4).

Class IV. Repeats from other genomic clones (see Fig. 1). It is unknown whether they are transcribed or not. These clones are the Y23 plasmid clone of Lifschytz and Hareven (1985), plasmid clones PY1 and PY9 (Vogt and Hennig 1989b), subclone 1.8 of lambda clone Ysla4 (Wlaschek et al. 1988) and lambda clone DhNo55 (Chapter 2). This class consists of 23 ay1 repeat sequences, including the original ay1 repeat (see Table 3 and Fig. 5).

The Ys1 repeat sequence data set. A fifth group of repeats consisted of repeats of the Ys1 family, which was initially described by Lifschytz et al. (1983), Lifschytz and Hareven (1985) and Lifschytz (1987) as the Y20 family. This family of Y-specific

repetitive DNA sequences was independently identified by Wlaschek et al. (1988), who introduced the name YsI, that is adopted here as well. YsI is derived from ay1 by a duplication of sequence positions 207 to 393 of ay1, and as shown by hybridizations to Southern blots of genomic DNA (Wlaschek et al. 1988; Chapters 2 and 4), it has the same sequence complexity as ay1. As a reference for a full-length repeat of the YsI family we used the 594 bp YsI R1 repeat that was sequenced by Wlaschek et al. (1988) (see Table 4 and Fig. 6). For the present analysis, we determined the sequences of several additional YsI repeats, which were subcloned as SalI fragments from the genomic clones DhNo255 and DhNo327 (Chapter 2). Altogether, 18 YsI repeats were used for the sequence alignments, containing a total of 6.8 kb of DNA (see Table 4 and Fig. 6).

DNA sequence alignments. For the analysis of DNA sequences, we used the software package of the University of Wisconsin Genetics Computer Group (Devereux et al 1984). Pairwise sequence alignments were done using the program LFASTA of Pearson and Lipman (1988). Multiple sequence alignments were done using the program PILEUP, with manual adjustments to increase sequence similarity.

Results

Any nucleotide position of ay1 can be deleted in members of the ay1 repeat family

The ay1 sequence originally described is an EcoRI DNA fragment of 393 bp (Vogt and Hennig 1986a). It was used as a reference in all sequence comparisons of ay1 repeats, since it represents the basic sequence complexity of the entire sequence family. We first determined for each individual ay1 repeat the nucleotide positions of the reference repeat present.

The results of these pairwise sequence alignments are presented in Figs. 2 to 5. In all figures, repeats that are tandemly organized within a cluster are grouped together. Of all 76 ay1 repeats analyzed, only 7, including ay1 itself, were full-length repeats without internal deletions. Full-length repeats occurred in all classes except in Class II of potentially transcribed repeats. In all classes, the majority of repeats was characterized by deletions, which vary in size from only a few nucleotides to more than half of the repeat length. The deletions did not occur at preferred positions, and also affected the ARS-like and enhancer-like sequence motifs described by Vogt and Hennig (1986a).

Several repeats shared identical deletions. Examples are repeats R2 and R3 of MY3 (Fig. 3) and repeats R6 and R9 of DhNo19 (Fig. 4). This most likely reflects the occurrence of successive sequence amplification events (Vogt and Hennig 1986b; Hennig et al. 1989). They must have occurred relatively recently, as any two repeats with identical deletions share 92-96% sequence similarity (not shown). This is much higher than between two randomly chosen repeats from the same class (see Table 5). Duplications of sequences within the repeat were found in only 4 cases: repeats R14 and R15 of DhNo19 (see Fig. 4), repeat R4 of YsIa and repeat R4 of DhNo55 (see Fig. 5).

For defining the beginning and the end of YsI repeats, we used as a reference the longest YsI repeat known, YsI R1 (see Fig. 6) (Wlaschek et al. 1988). It has a length of 594 bp. Of the 18 YsI repeats analyzed, only the reference repeat

Table 1. Possibly transcribed ayl repeats (Class II)

repeat name	length (bp)	nucleotide position	EMBLaccession number
MY3[1] R1	153	1188-1340	X04811
MY3 R2	143	1341-1483	X04811
MY3 R3	156	1484-1639	X04811
MY3 R4	334	1640-1973	X04811
MY3 R5	356	1974-2329	X04811
DhNo86 R1	241	193-433	X74540
DhNo90 R1	112	545-656	X74536
DhNo90 R2	120	750-869	X74536
DhNo90 R3	316	73-388	X74537
DhNocos6 R1	271	19-289	X74884
DhNocos6 R2	80	290-369	X74884
DhNocos6 R3	265	1-265	X74885
DhNocos6 R4	238	266-503	X74885
DhNocos6 R5	46	504-549	X74885
DhNocos6 R6	282	1-282	X74886
DhNocos6 R7	383	283-665	X74886
DhNocos6 R8	303	666-968	X74886
DhNocos6 R9	377	969-1345	X74886
DhNocos6 R10	54	1346-1399	X74886

Note: [1]The complete sequence of MY3 (2502 bp) has been published by Vogt and Hennig (1986b)

Table 2. Nontranscribed ayl repeats (Class III)

repeat name	length (bp)	nucleotide position[1]
DhNo19 R1	157	763-1919
DhNo19 R2	265	1920-2184
DhNo19 R3	163	2185-2347
DhNo19 R4	37	3013-3049
DhNo19 R5	357	3050-3406
DhNo19 R6	329	3407-3735
DhNo19 R7	285	3736-4020
DhNo19 R8	191	6675-6865
DhNo19 R9	333	6866-7198
DhNo19 R10	343	7199-7541
DhNo19 R11	394	7542-7935
DhNo19 R12	329	7936-8264
DhNo19 R13	273	8265-8537
DhNo19 R14	370	15542-15911
DhNo19 R15	471	15912-16382
DhNo19 R16	78	16393-16470

Note: [1]The complete sequence of DhNo19 has been deposited in the EMBL database under accession number X74538

Table 3. ay1 repeats from clones without *gypsy* (ClassIV)

repeat name	length (bp)	nucleotide positions	EMBL accession number
ay1	393	1-393	X04812
by9[1]	532	1-532	X05043
dy9[1]	491	1-491	X05047
gly9	464	1-464	-[2]
g3y9	522	1-522	-[2]
Ysla R1[3]	375	RC[4] of 1620-1994	M24760
Ysla R2	367	RC of 1253-1619	M24760
Ysla R3	388	RC of 865-1252	M24760
Ysla R4	468	RC of 397-864	M24760
Ysla RS	390	RC of 7-396	M24760
Y23Ns	240	1-240	-[5]
Y23BNs R1	249	1-249	-[6]
Y23BNs R2	187	304-490	-[6]
Y23BNs R3	200	491-696	-[6]
DhNo55 R1	332	1-332	X74887
DhNo55 R2	205	333-537	X74887
DhNo55 R3	296	1-296	X74888
DhNo55 R4	270	297-566	X74888

Notes:
1 described by Vogt and Hennig (1986a)
2 see Vogt and Hennig 1986a for more details
3 described by Wlaschek et al. (1988)
4 Reverse Complement
5 described by Lifschytz and Hareven (1985)
6 Y23BNs is an unpublished, 696 bp-long sequence made available by E. Lifschytz

Table 4. Origin of YsI repeat sequences

repeat name	length (bp)	nucleotide positions	EMBL accession number
YsI R1	594	RC[2] of 7-600	M24761
YsI R2	542	RC of 601-1142	M24761
Y20f1	516	1-516	-[3]
Y20f2	497	1-497	-[3]
Y20Ns	240	1-240	-[4]
25SPROBE	562	1-562	X75056
25SS7	530	1-530	X75055
25SS6	514	1-514	X75057
25SS2	508	1-508	X75058
25SS1	504	1-504	X75059
25SS4	413	1-413	X75060
25SS12	329	1-329	X75061
25SS10	165	1-165	X75062
25SS13	119	1-119	X75063
25SS23	97	1-97	X75064
25SS16	68	1-68	X75065
327S4	394	1-394	X75066
327S2	248	1-248	X75067

Notes:
1 described by Wlaschek et al. (1988)
2 Reverse Complement
3 described by Lifschytz (1987)
4 described by Lifschytz and Hareven (1985)

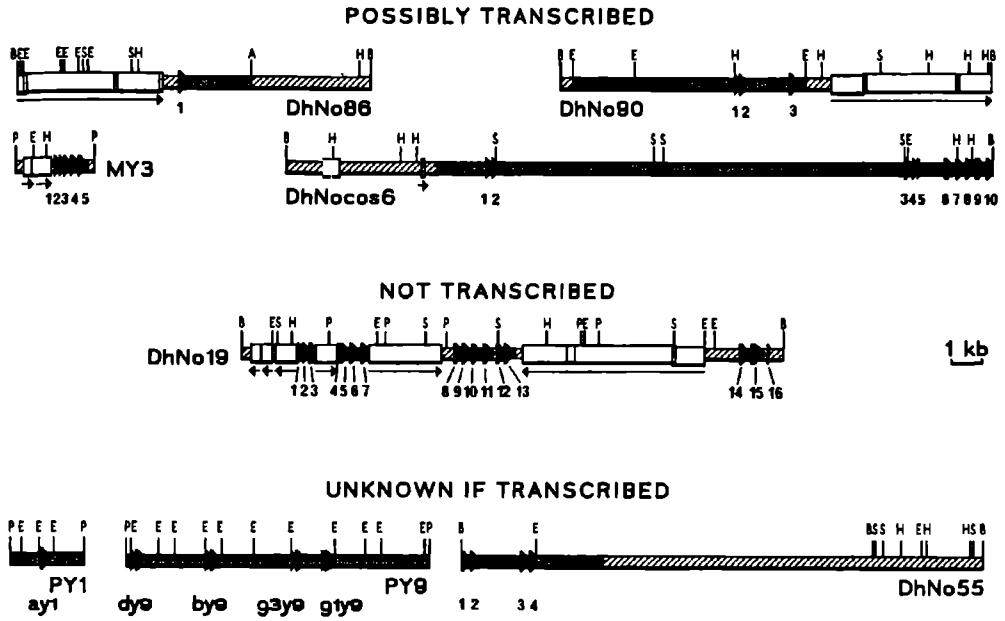
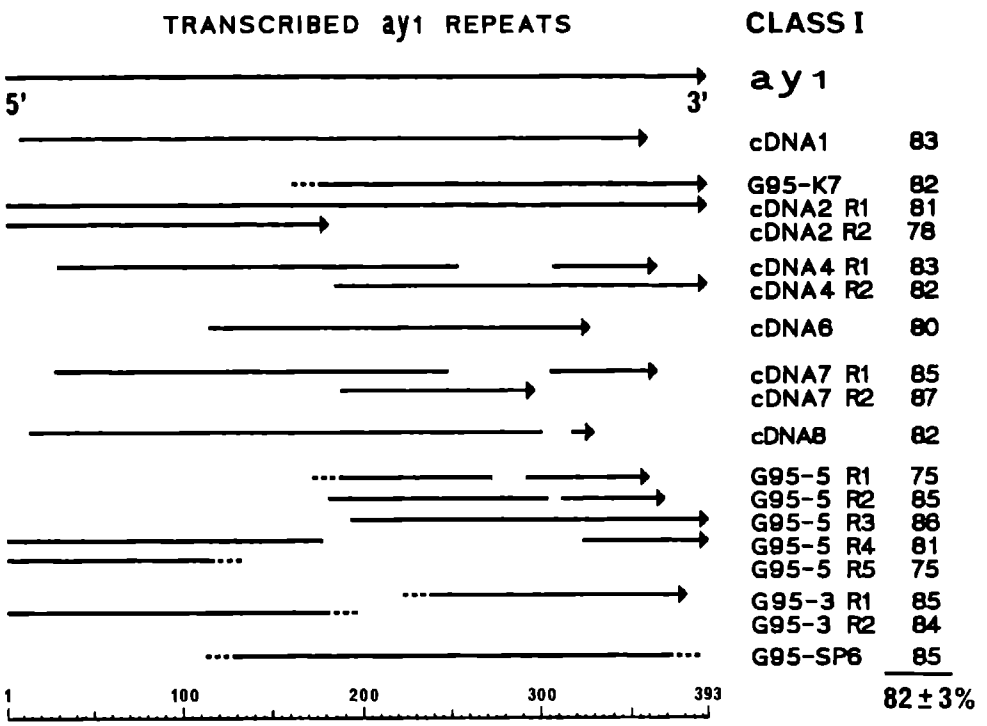
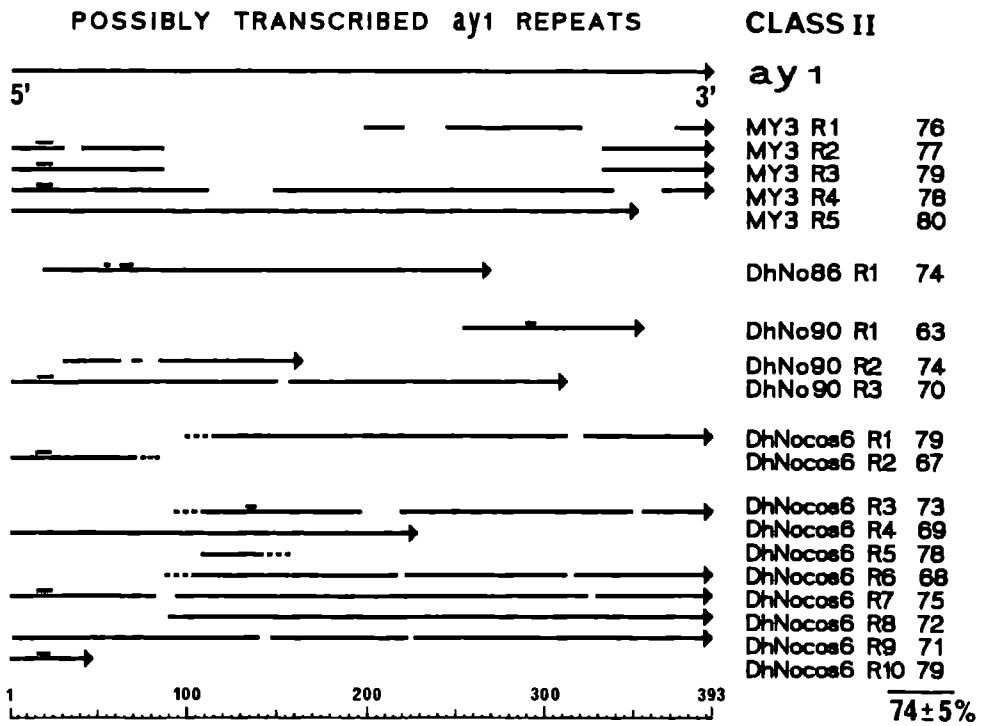


Fig. 1. Position of ay1 repeat sequences of Classes II, III and IV in genomic clones from the *Y* chromosome In all restriction maps, individual ay1 repeats, as identified by sequence alignment with the 393 bp reference repeat of Vogt and Hennig (1986a), are indicated as black arrowheads, with the arrowhead pointing in the direction of transcription Restriction fragments harboring ay1 repeats are indicated by dark shading Class I ay1 repeats are transcribed from the same strand of DNA as adjacent *gypsy* sequences (indicated as white rectangles, with the arrow indicating *gypsy* transcription) These clones are therefore "possibly transcribed Class II ay1 repeats are surrounded by *gypsy* sequences which have both orientations relative to ay1 Class III ay1 repeats are from clones without *gypsy* sequences In all clones, restriction fragments that hybridize neither to ay1 nor *gypsy* are indicated by diagonal hatching Restriction sites *A*, AvaI, *B*, BamHI, *E*, EcoRI, *H*, HindIII, *P*, PstI, *S*, SalI

Fig. 2 (facing page, upper figure). Alignment of transcribed ay1 repeats of Class I with the ay1 reference repeat defined by Vogt and Hennig (1986a) These repeats were derived from cDNA clones or their genomic clone of origin by Papenbrock (1991) In all repeats, the arrowhead indicates the direction of transcription For several repeats the 5' or 3' end, or both, were not determined (indicated by stippled ends of the repeats) In all other cases, the 5' end of each ay1 repeats was defined by the 3' end of a preceeding repeat, and the 3' end was defined by the 5' end of a following repeat. All deletions larger than two nucleotides are indicated The scale bar at the bottom indicates nucleotide positions The numbers at the right are the sequence similarity to the ay1 reference repeat at the top of the figure, the number at the bottom is the average similarity and the standard deviation

Fig. 3 (facing page, lower figure). Alignment of possibly transcribed ay1 repeats of Class II For explanantion, see Fig 2, with the addition that the lines above several of the repeats indicate small insertions The 5' end of each repeat was determined either by the 3' end of a preceeding repeat, or by the 3' end of an upstream located, unrelated sequence The 3' end of each repeat was determined either by the 5' end of a following repeat, or by the 5' end of a downstream located, unrelated sequence For the origin of the ay1 repeat sequences, see Table 1


TRANSCRIBED ay1 REPEATS
CLASS I
ay1
5'
3'
cDNA1 83
G95-K7 82
cDNA2 R1 81
cDNA2 R2 78
cDNA4 R1 83
cDNA4 R2 82
cDNA6 80
cDNA7 R1 85
cDNA7 R2 87
cDNA8 82
G95-5 R1 75
G95-5 R2 85
G95-5 R3 86
G95-5 R4 81
G95-5 R5 75
G95-3 R1 85
G95-3 R2 84
G95-SP6 85
82 ± 3%
1
100
200
300
393


POSSIBLY TRANSCRIBED ay1 REPEATS
CLASS II
ay1
5'
3'
MY3 R1 76
MY3 R2 77
MY3 R3 79
MY3 R4 78
MY3 R5 80
DhNo86 R1 74
DhNo90 R1 63
DhNo90 R2 74
DhNo90 R3 70
DhNocos6 R1 79
DhNocos6 R2 67
DhNocos6 R3 73
DhNocos6 R4 69
DhNocos6 R5 78
DhNocos6 R6 68
DhNocos6 R7 75
DhNocos6 R8 72
DhNocos6 R9 71
DhNocos6 R10 79
74 ± 5%
1
100
200
300
393

was full-length. All 17 other repeats contained deletions varying in size from 10 nucleotides to two-thirds of the entire repeat length. Again, no nucleotide was deleted more often than others, and any nucleotide was found to be deleted in at least one repeat.

Thus, within both the ayl and the YsI family, repeats without deletions are rare. The deletions randomly affect any position within the repeat sequence.

Sequence heterogeneity within transcribed and nontranscribed repeats

We determined for each ayl repeat class the percentage of sequence similarity with the ayl reference repeat (Figs. 2 to 5). The transcribed repeats of Class I had the highest average percentage of sequence similarity to ayl (82%, standard deviation 3%), but it was slightly lower compared to the average similarity of the YsI repeats with the YsI R1 reference repeat (86%, standard deviation 4%).

We also determined the sequence heterogeneity among the transcribed and nontranscribed ayl repeats by performing all possible pairwise sequence comparisons between the repeats of a given class. The results of this analysis are summarized in Table 5. Among the repeats of the ayl family, the highest average sequence similarity was found between the Class I sequences derived from cDNA clones (83%, standard deviation 4%). This however, is within the range also displayed by the nontranscribed ayl repeats of Class III (79%, standard deviation 6%),

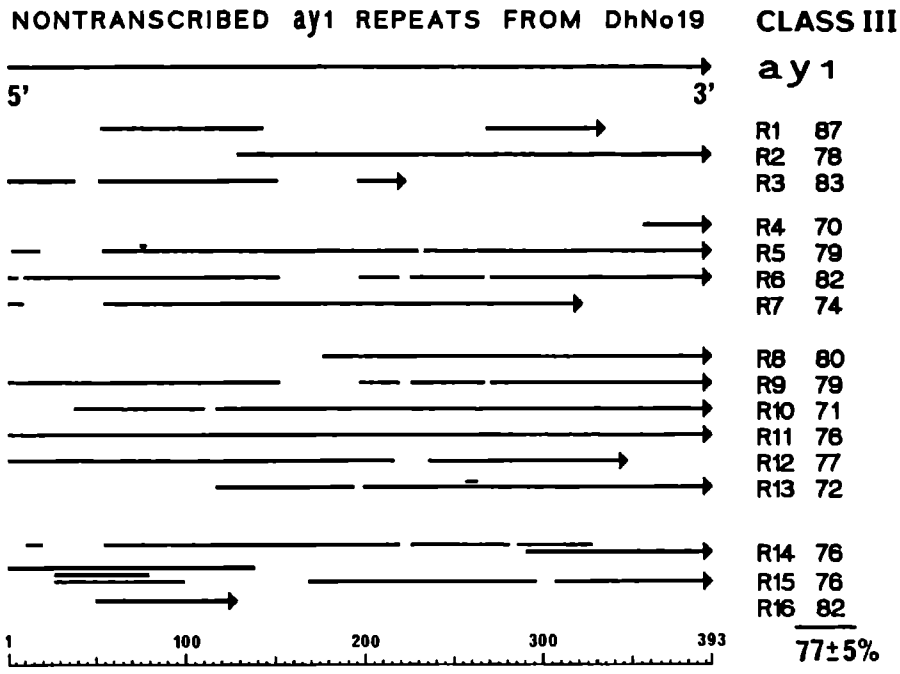
Table 5. Heterogeneity in the ayl and YsI families of repetitive DNA sequences

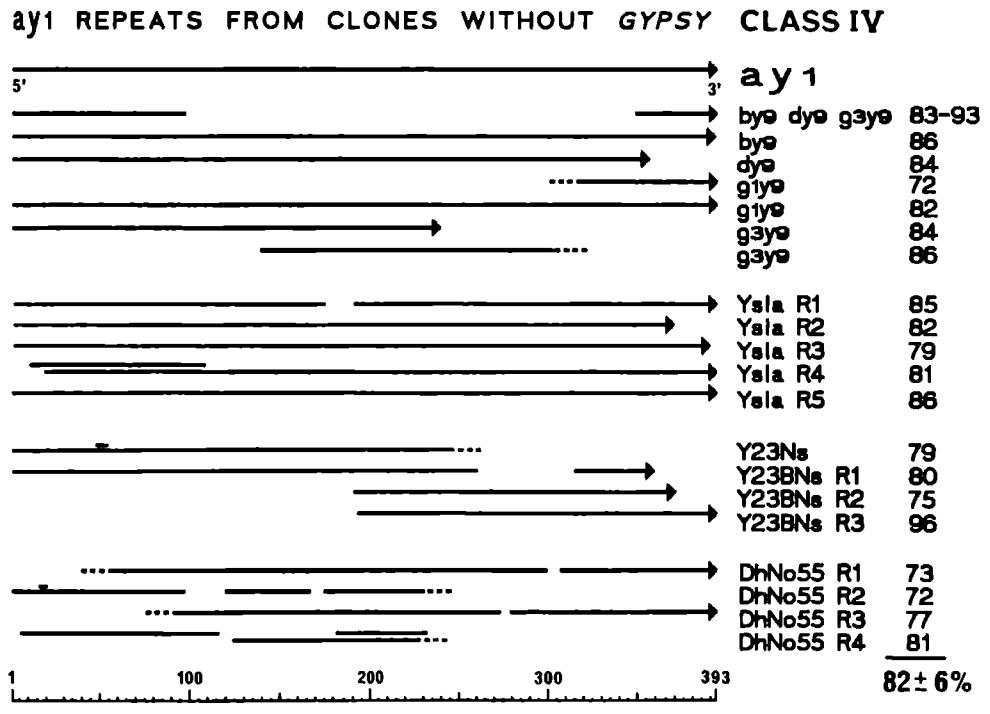
repeat class	number of repeats analyzed	number of pairwise alignments[1]	average % of sequence similarity ± SD[2]	spread (%)
ayl Class I	18	129	83 ± 4	71-96
ayl Class II	19	145	75 ± 8	60-96
ayl Class III	17[3]	123	79 ± 6	66-96
ayl Class IV	23	n.d.[4]	n.d.	n.d.
YsI	14[5]	89	85 ± 4	75-99

Notes: [1] number of all possible alignments between two repeats of the same class
[2] Standard Deviation
[3] Because of the duplication in repeat 15 of DhNo19 (Fig. 4), the sequence was split into two parts that were analyzed separately
[4] not determined
[5] The short repeats 25SS10, 25SS13, 25SS16 and 25SS23 were not included

Fig. 4 (facing page, upper figure). Alignment of nontranscribed ayl repeats of Class III. For explanation, see Figs. 2 and 3. Repeats 14 and 15 contain sequence duplications and triplications, indicated by the double and triple lines, respectively. For the origin of the ayl repeat sequences, see Table 2

Fig. 5 (facing page, lower figure). Alignment of Class IV ayl repeats from clones without *gypsy* sequences. For explanation, see Figs. 2, 3 and 4. For the origin of the ayl repeat sequences, see Table 3


NONTRANSCRIBED ay1 REPEATS FROM DhNo19
CLASS III
5'
3'
ay1
R1 87
R2 78
R3 83
R4 70
R5 79
R6 82
R7 74
R8 80
R9 79
R10 71
R11 76
R12 77
R13 72
R14 76
R15 76
R16 82
1
100
200
300
393
77±5%


ay1 REPEATS FROM CLONES WITHOUT GYPSY
CLASS IV
5'
3'
ay1
bye dye g3ye 83-93
bye 86
dye 84
g1ye 72
g1ye 82
g3ye 84
g3ye 86
Ysla R1 85
Ysla R2 82
Ysla R3 79
Ysla R4 81
Ysla R5 86
Y23Ns 79
Y23BNs R1 80
Y23BNs R2 75
Y23BNs R3 96
DhNo55 R1 73
DhNo55 R2 72
DhNo55 R3 77
DhNo55 R4 81
1
100
200
300
393
82±6%

as well as within that of the nontranscribed YsI repeats (85%, standard deviation 4%). Class II ay1 repeats are most heterogeneous, displaying the lowest average sequence similarity (75%), with the largest standard deviation (8%).

The duplicated parts within repeats R14 and R15 of DhNo19, within repeat R4 of YsIa, and within repeat R4 of DhNo55 were not found to be more similar to one another than to the corresponding regions of other repeats. As an example, Fig. 7 shows the duplicated sequences in repeats R14 and R15 of DhNo19. The duplicated parts within one sequence share 70-75% similarity with each other, but this is not higher than the similarity between two randomly chosen repeats from this clone. Thus, following their duplication, the duplicated sequences started to accumulate point mutations.

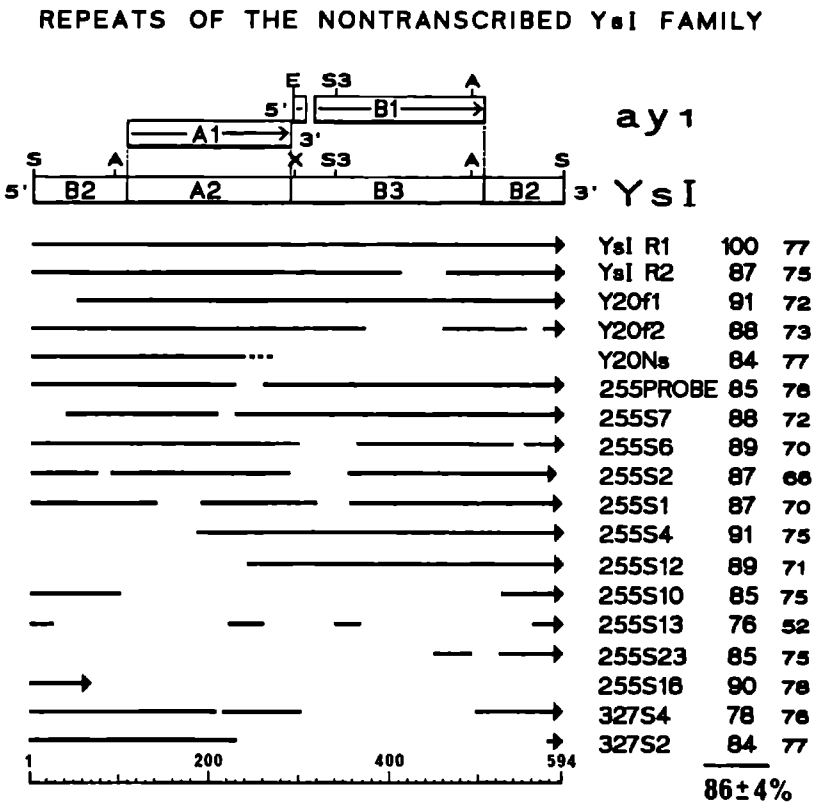


Fig. 6. Alignment of repeats of the YsI family. As a reference repeat for sequence alignments we used the YsI R1 repeat of Wlaschek et al. (1988). For the origin of the YsI repeat sequences, see Table 4. The beginning and the end of each YsI repeat are defined by the SalI site used for cloning. The numbers on the right indicate the sequence similarity to YsI R1 (large numbers) and to the ay1 reference repeat (small numbers). In the upper part of the figure the ay1 and YsI reference repeats are aligned, revealing the underlying substructure of both related families of repetitive DNA sequences. The numbering of the A and B blocks is according to Wlaschek et al. (1988). The arrows in the ay1 repeat sequence indicate the orientation of transcription. Restriction sites which frequently occur in repeats of each family are indicated. Abbreviations are *A*, AluI, *E*, EcoRI, *S*, SalI, *S3*, Sau3A and *X*, XbaI

In summary, the sequence comparisons imply that the transcribed ayl repeats are not more conserved than nontranscribed ayl repeats. The homogeneity among the transcribed ayl repeats is not higher than between the nontranscribed repeats of the YsI family.

Comparison of consensus sequences of transcribed and nontranscribed ayl repeats

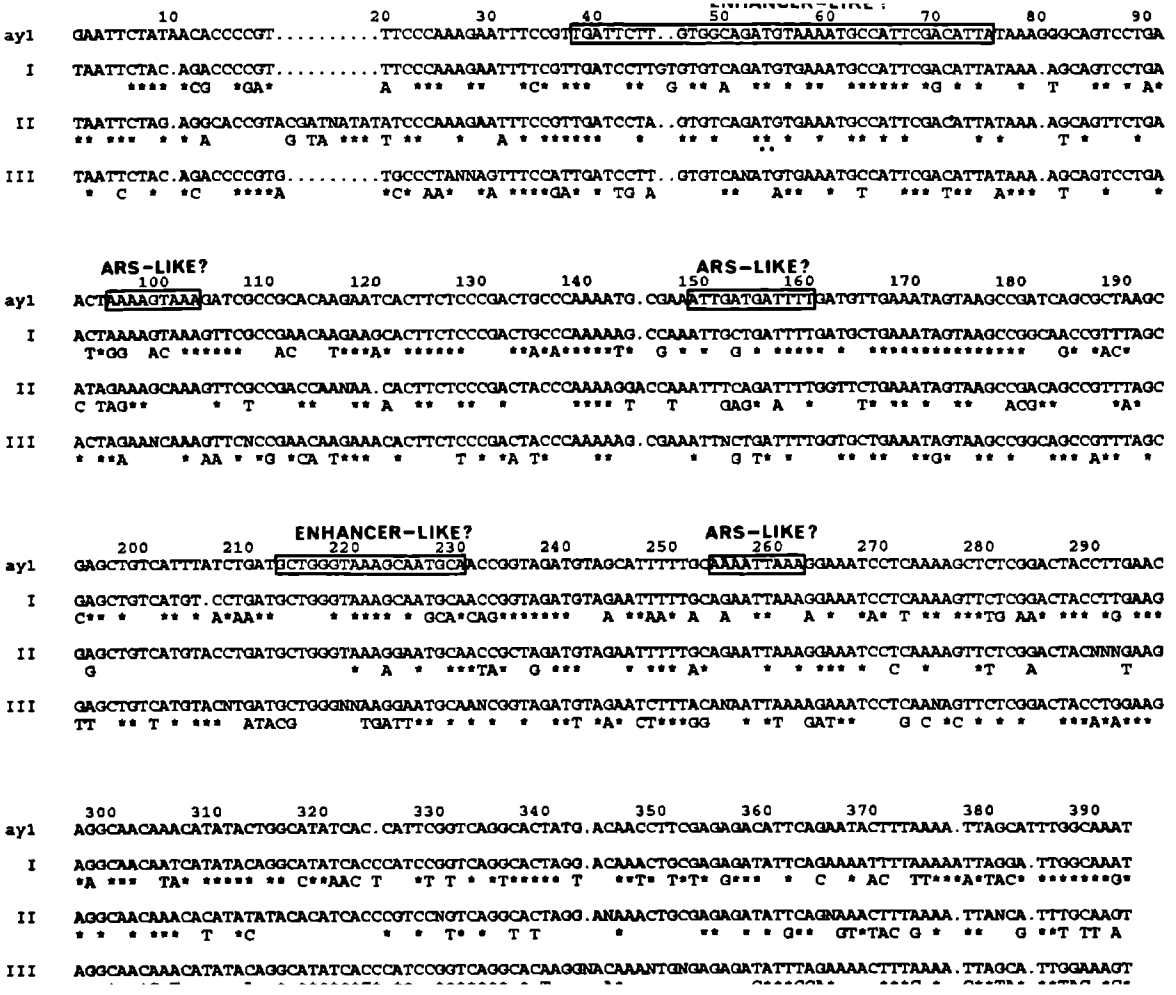
Although any nucleotide of the transcribed ayl repeats may be deleted, this does not exclude that certain sequences are conserved between all transcribed repeats. We therefore used the program PILEUP for performing multiple sequence alignments between all repeats of the Classes I, II and III, respectively. For each class, a consensus sequence could be derived.

The three ayl consensus sequences are shown in Fig. 8, where they are aligned together with the ayl reference repeat sequence of Vogt and Hennig (1986a). Only 47% of all sequence positions were occupied by the same nucleotide in all transcribed repeats of Class I, but their distribution was completely random. The longest uninterrupted array of conserved nucleotides had a length of 15, corresponding to positions 166-180 of ayl (Fig. 8), but these sequence positions were not conserved in the potentially transcribed Class II repeats. As already indicated

```
      ayl    291 TTGAACAGGCAACAAACATATACTG-GCATATCA-CCATT 328

DhNo19R14 15773 TGGAAGAAGCAACAAACATATAAGGCACAT-CCACCCATC 15810
DhNo19R14 15811 TGGAAGAGGCAAGAAACGTATAAGG-GTATATCACCCATC 15849
                ******* **** **** *******   **  ** ****

      ayl     27 AGAATTTCCGTTGATTCTTGTGGCAGATGTAAAATGCCATTCGACATTATAAA 79

DhNo19R15 15937 ATAATTTCCATTGATCCTATTGTCAAATGTGAAATGCCTTTCGACATCATA-- 15987
DhNo19R15 16045 AAAAGATACGTTGATCCTAGTGTCAGATG--AAATGTCATTCGCCATTATGAA 16095
DhNo19R15 16096 AATACGATATATGTTCCTAGTGTCAGACGTGAAATGATATTCGACATTATAAA 16148
                *  *       ** ***** ***** * *  *****   **** *** **
```

Fig. 7. Sequence alignment of the duplicated and triplicated sequences in repeats 14 and 15 of DhNo19 and the ayl reference repeat of Vogt and Hennig (1986a). Nucleotides shared between the duplicated sequences are indicated by an asterisk(*). Gaps, which were introduced to increase similarity, are indicated by dots

Fig. 8 (next page). Comparison of consensus sequences for ayl repeats of Classes I, II, and III. The original ayl repeat of Vogt and Hennig (1986a) is also included in the alignment. In each consencus sequence, nucleotides that are conserved in all the members of a particular class, are indicated by *asterisks* (*). Positions occupied by the same nucleotide in all but one repeat are indicated by a *single letter*. Positions preferentially occupied by two nucleotides are indicated by *two letters*, the less frequently occurring nucleotide being written below the more frequently occurring one. Positions not preferentially occupied by a given nucleotide are indicated by the letter *N*. Gaps introduced to increase the similarity between the different consensus sequences are indicated by *dots*. *Numbers* refer to the ayl reference repeat. In this repeat, the sequence motifs with similarity to the ARS consensus sequence of *Saccharomyces cerevisiae* or to eukaryotic enhancers, as proposed by Vogt and Hennig (1986a), are indicated. The Class I consensus is based on 18 repeats (see Fig. 2), the Class II consensus on 19 repeats (see Fig. 3), and the Class III consensus on 16 repeats (see Fig. 4)


ENHANCER-LIKE?
ARS-LIKE?
ARS-LIKE?
ENHANCER-LIKE?
ARS-LIKE?
ay1
I
II
III

by their larger heterogeneity, the Class II repeats only had 25% of all nucleotide positions conserved, which is less than the 36% found for the nontranscribed Class III repeats. Of the 47% conserved sequence positions in Class I repeats, less than half were also conserved in all Class II repeats. The fraction of nucleotide positions conserved in all 53 repeats of the three classes was only 8%. The putative ARS-like and enhancer-like sequences, as described by Vogt and Hennig (1986a), were not better conserved compared to other sequence positions, even not in the transcribed Class I repeats.

Sequences potentially interfering with the continuity of transcription within the loops are likely to be absent in transcribed repeats. We did not find the highly conserved polyadenylation signal 5' AATAAA 3' (Manley 1988) in the transcribed Class I repeats, whereas this sequence was present in five repeats of the nontranscribed Class III. Since both classes comprised almost equal numbers of repeats, together containing comparable amounts of DNA, this finding suggests that polyadenylation signals are selected against in the transcribed ayl repeats.

In summary, all ayl repeats differ considerably at the DNA sequence level. Almost half of all the sequence positions in the repeats derived from cDNA clones are occupied by the same nucleotide, but we have no indications that any segment of the ayl sequence is better conserved than other segments. The large extent of sequence heterogeneity among the members of the ayl family suggests that all repeats undergo random changes in nucleotide composition, irrespective of whether they are transcribed in the *Nooses* loop pair.

Discussion

The present analysis of ayl repeat sequences was conducted to investigate whether, at the level of the primary DNA sequence, the ayl repeats that are transcribed in the *Nooses* are different from those that are not transcribed. Assuming that the transcribed repeats are under some sort of selective pressure, whereas the repeats located outside of the loop are not, such an analysis might reveal conserved sequence motifs in the transcribed repeats that might be important for the function of the loop-forming fetility gene.

Two observations lead to the conclusion that the transcribed ayl repeats are not under a selective constraint that does not act on nontranscribed repeats. First, any position within ayl can be deleted, irrespective of whether the affected repeat is transcribed or not. Second, although there are some conserved nucleotides in the transcribed repeats, the transcribed repeats are not significantly better conserved than ayl repeats that are not transcribed or than repeats of the nontranscribed Ysl family.

Thus, the members of the ayl repeat are subject to random mutations and deletions. The average sequence similarity between the ayl repeats is less than that between the members of the YLII and *rally* families of repetitive DNA sequences that are transcribed in the loop pair *Threads* of *D. hydei* (Huijser and Hennig 1987; Wlaschek et al. 1988; Trapitz et al. 1988, 1992; Trapitz 1992). Transcribed members of the YLII family, which have a sequence complexity of 76 bp,

share 95% sequence similarity, as shown by the analysis of more than 25 repeats by Papenbrock (1991). Sequences of *rally* repeats derived from cDNAs are not available, but the 6 repeats described by Huijser and Hennig (1987), with a sequence complexity of 200 bp, display 90-98% sequence similarity. Furthermore, most of the transcribed repeats of the YLII family are full-length, in contrast to those of the ay1 family, which all suffered from deletions (Papenbrock 1991). Also most, if not all, members of the *rally* family are full-length, as Southern blots of genomic DNA reveal a regular periodicity of 200 bp (Huijser and Hennig 1987).

We assume that these differences in the conservation of the transcribed repeats, both in repeat size and sequence, reflect differences in the evolutionary age of the lampbrush loop pairs. It would appear, therefore, that the *Threads* are of a more recent origin than the *Nooses*. This assumption is supported by other observations. Cytological and genetic studies of I. Hennig (1978,1982) indicate that the *Y* chromosome of *D. neohydei*, a sibling species of *D. hydei*, carries a fertility gene that forms a *Threads*-like loop pair. This gene is able to complement a deficiency of fertility gene *A* of *D. hydei*, that forms the *Threads* loop pair. The more distantly related species *D. eohydei* lacks a loop pair with a *Threads*-like morphology. Both species however, have a *Nooses*-like loop pair as judged from cytogenetic (I. Hennig 1982) and molecular observations (Vogt et al. 1986; also see Chapter 4). Together with *D. hydei*, these two closely related species from the *hydei* subgroup are the only species of *Drosophila* carrying repeats of the ay1 and YLII families (Vogt et al. 1986; Hareven et al. 1986; Wlaschek et al. 1988), but, again documenting the relatively recent origin of the *Threads* loop pair, the *rally* family is present only in *D. hydei* (Huijser and Hennig 1987). Thus, the loop-forming genes seem to evolve by the sudden transcriptional activation and amplification of certain arrays of tandemly organized repetitive DNA sequences. Subsequently, individual repeats accumulate point mutations and deletions.

The ay1 repeats are not the only repetitive DNA sequences transcribed in the *Nooses* loop pair that are randomly degenerating. This has also been shown for the second major sequence constituent of the loop, the defective and truncated retrotransposons of the *gypsy* family (see Chapters 5 and 7). Both sequences, ay1 and *gypsy*, together account for at least 80-90% of the DNA that is transcribed in the *Nooses* (see Chapters 2, 5 and 7). Thus, it is highly unlikely that additional base pair substitutions and deletions in the primary DNA sequences of these major loop components will affect the function of fertility gene *Q*. The only possible exception may be that polyadenylation signals are not permitted in the transcribed sequences. We have shown that *Nooses* transcripts containing ay1 repeats are not polyadenylated (Chapter 5). In addition, since all repetitive loop constituents identified so far have the same orientation within the loop-forming transcription units (see Chapters 2 and 5 and references therein), it also seems that the reversal of polarity of such sequences is selected against as well.

In conclusion, the detailed sequence analysis of the major loop constituents of the *Nooses* loop pair strongly suggests that other sequences, in addition to ay1 and *gypsy*, represent the target for mutations that inactivate the associated

fertility gene *Q*. Further implications of these findings for models of the molecular organization of the loop-forming fertility genes and for hypotheses regarding their function and evolution are discussed in Chapter 9.

Acknowledgements. We are indebted to Prof Dr. Eliezer Lifschytz, Dr. Thomas Papenbrock and Prof. Dr. Hans Bünemann for communicating unpublished DNA sequence data, and to Monique Wilbrink for assisting us with the calculations and sequence alignments. Wieli Janssen supported us with expert technical assistance.

CHAPTER 7

Degenerating *gypsy* retrotransposons in a male fertility gene on the *Y* chromosome of *Drosophila hydei*

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Abstract. During the evolution of the *Y* chromosome of *Drosophila hydei*, retrotransposons became incorporated into the lampbrush loop pairs formed by several of the male fertility genes on this chromosome. Although insertions of retrotransposons are involved in many spontaneous mutations, they do not affect the functions of these genes. We have sequenced *gypsy* elements that are expressed as constituents of male fertility gene *Q* in the lampbrush loop pair *Nooses*. We find that these *gypsy* elements are all truncated and specifically lost those sequences that might interfere with the continuity of lampbrush loop transcription. Only defective coding regions are found within the loop. *Gypsy* is not transcribed in loops of many other *Drosophila* species harboring the family. These results suggest that any contribution of *gypsy* to the function of male fertility gene *Q* does not depend on a conserved DNA sequence. We propose a general model for the evolution of the loop-forming fertility genes, explaining how *gypsy* became a loop constituent without interfering with the function of gene *Q*.

Introduction

Many eukaryotes have morphologically distinct sex chromosomes. They are often assumed to have evolved from a pair of genetically and morphologically identical chromosomes (Ohno 1967; Charlesworth 1991). If meiotic recombination is suppressed over all or most of the length of a sex chromosome, sex-specific determinants may remain restricted to one of the homologous chromosomes. Muller (1918) was the first to point out that in such a genetic situation the sex chromosome which is present only in the heterogametic sex will tend to accumulate deleterious mutations, leading to a loss of genetic activity of this chromosome. This however, would interfere with normal gene expression as the appropriate gene dosage is critical for many metabolic processes. Several models have been proposed to explain the degeneration of the *Y* chromosome and the concomitant evolution of a dosage compensation mechanism for genes on the *X* chromosome (Charlesworth 1978; Rice 1987).

In *Drosophila* males, there is no meiotic recombination (Morgan 1912,1914). However, despite of the absence of *Y*-linked genes essential for male viability, the *Drosophila* Y chromosome is not a degenerated, inert *X* chromosome. In most *Drosophila* species, the *Y* chomosome carries genes that are essential for male fertility, as shown first for *D. melanogaster* by Bridges (1916). Six male fertility genes have been mapped on the *Y* chromosome of this species and at least seven on that of *D. hydei* (reviewed by Hackstein 1987; Hennig 1985, 1990; Gatti and Pimpinelli 1992). In both species several of these genes form lampbrush loops during meiotic prophase, that are seen as prominent structures in the nuclei of primary spermatocytes (Hess and Meyer 1968). The loops are large transcription units with sizes up to 1500 kb of DNA (Grond et al. 1983; de Loos et al. 1984).

Each lampbrush loop-forming fertility gene corresponds to one complementation group. Deletions or mutations of any individual loop-forming gene lead to an arrest during the final stages of sperm development (Hess and Meyer 1968; Hardy et al. 1981; Hackstein et al. 1982, 1991). Therefore, each loop-forming gene performs

a unique function that is indispensable for fertility. Deletion or mutation of the loop-forming gene *kl-5* on the *Y* chromosome of *D. melanogaster* results in the absence of the outer dynein arms in each peripheral microtubule doublet in the sperm axoneme (Hardy et al. 1981), and the structural gene for a dynein β-heavy chain isoform has been assigned to this gene (Goldstein et al. 1982; Gepner and Hays 1993). Although temperature-sensitive alleles have been isolated for several of the loop-forming genes on the *D. hydei Y* chromosome (Leoncini 1977) we have no direct evidence for a protein-coding function of any of the fertility genes in this species.

On the contrary, our studies of the molecular composition of these genes have revealed that the DNA transcribed in the loops consists of two major types of repetitive sequences (Vogt et al. 1982; Hennig et al. 1983; Vogt and Hennig 1983; 1986a,b; Huijser and Hennig 1987; Huijser et al. 1988; Chapter 2). The first type are satellite-like *Y*-specific DNA sequences without protein coding capacity, that have also been studied by others (Lifschytz et al. 1983; Wlaschek et al. 1988; Trapitz et al. 1988, 1992). The best characterized example is the ayl family (Vogt et al. 1982; Vogt and Hennig 1986a), which is transcribed in fertility gene *Q*, forming the lampbrush loop pair *Nooses*. The second type of lampbrush loop components are sequences that also occur on other chromosomes, and which are therefore called *Y*-associated (Vogt and Hennig 1986b). In at least three of the five loop-forming genes of *D. hydei* retrotransposons have been identified as *Y*-associated loop constituents. Members of the *micropia* family are transcribed in the fertility genes forming the loop pairs *Threads* and *Pseudonucleolus* (Huijser et al. 1988; S. Lankenau et al. 1994), and members of the *gypsy* family in the *Nooses* loop pair (Chapter 5). The *gypsy* sequences occur interspersed between the *Y*-specific loop constituents, the ayl repeats.

There are theoretical and experimental indications that self-replicating, transposable elements accumulate in regions of the genome not subjected to meiotic exchange (Charlesworth and Langley 1991). Since there is no recombination in *Drosophila* males, it is not unusual to find retrotransposons on the *Y* chromosome. However, our finding that they are constituents of *Y*-linked fertility genes is quite unexpected, since in *D. melanogaster* the insertion of a retrotransposon has been shown to frequently cause spontaneous mutations. Especially *gypsy* seems to be involved in a disproportionately large number of mutations (Green 1988; Bingham and Zachar 1989). Moreover, recent studies of the larval cuticle protein genes on the neo-*Y* chromosome of *D. miranda* even suggest that retrotransposon insertion is a specific mechanism for the destruction of genetic activity, leading to the evolution of a *Y* chromosome (Steinemann and Steinemann 1991, 1992; Steinemann et al. 1993).

Thus, the identification of retrotransposons as natural constituents of *Y*-linked fertility genes seems to contradict Muller's (1918) hypothesis of sex chromosome evolution, and raises additional questions. Why do these retrotransposons fail to cause male sterility? Do they encode functional proteins? How did they become incorporated into the fertility genes without causing sterility? Are they important for fertility gene function? In this paper we address these questions by performing a detailed analysis of *gypsy* sequences from male fertility gene *Q* of *D. hydei*.

Materials and Methods

Drosophila strains. Individuals of *D. hydei* (*Tübingen* wild-type strain), *D. melanogaster* (*Oregon-R* wild-type strain) and also of wild-type strains of *D. repleta* and *D. virilis* were taken from our laboratory collection. Flies were kept at 18°C as described (Hackstein et al. 1982).

Genomic clones from the D. hydei Y chromosome. The sequence of clone MY3 was described earlier by Vogt and Hennig (1986b), and that of DhMiF8 by Huijser et al. (1988) and D.-H. Lankenau et al. (1990). All other clones are described in Chapter 2. Using ay1 sequences as a probe, these clones were isolated either from a BamHI genomic library in lambda vectors (for example DhNo19) or from a BamHI library in cosmid vectors (for example DhNocos6).

DNA sequence analysis. For DNA sequencing, restriction fragments from lambda or cosmid clones were either directly subcloned in M13mp18 or M13mp19 or, before cloning in M13, first subcloned in the pGEM3 plasmid vector for more detailed restriction mapping. Subcloning was as described (Chapter 2). DNA sequences were determined using the dideoxy chain-termination method as described by Amersham (1984).

Analysis of DNA sequences was performed using the program set of the University of Wisconsin Genetics Computer Group (Devereux et al. 1984). The *gypsy*-like sequences were detected by database searches, using the FASTA program of Pearson and Lipman (1988). For determining the overall level of similarity between two DNA sequences, we calculated the percentage positional identity from a sequence alignment generated by LFASTA (Pearson and Lipman 1988). If the two sequences could be aligned only by allowing deletions and insertions, the corresponding nucleotide positions were excluded from the calculation. The alignment of long terminal repeat (LTR) sequences was made using the program PILEUP, with manual adjustments. Positions of ay1 repeats were defined using the original 393 bp ay1 repeat described by Vogt and Hennig (1986a) as a reference. For determining the amino acid similarity between the putative proteins predicted from the *gypsy* open reading frames (ORFs), corrections for frame shifts and stop codons were introduced in the *D. hydei* ORFs.

D. melanogaster transposable element probes. For testing whether *D. hydei* and *D. melanogaster* shared families of transposable elements other than *gypsy* and *micropia*, we first surveyed the literature and then collected probes for those families of *D. melanogaster* not known to be present in *D. hydei*. The following families were tested: *17.6*, *3S18*, *B104*, *Springer* and *FB4* (provided by D.-H. Lankenau from a laboratory collection of V.G. Corces), *doc* and *pogo* (provided by K. O'Hare), *BS*, *mdg3*, and *HMS Beagle* (provided by A. Kim), *I* (provided by D.J. Finnegan) and *1731* (provided F. Fourcade-Peronnet). We also tested the *tom* element from *D. ananassae* (provided by S. Tanda). With the exception of *tom*, descriptions of all elements can be found in a compilation by Finnegan (1990).

Of all these families of transposable elements, only *17.6* and *B104* were found to be present in *D. hydei*. We give a more detailed description only for these probes. For *17.6* we used an internal ClaI fragment corresponding to nucleotide positions 387 to 7313 of the 7439 bp *17.6* element (Saigo et al. 1984). For *B104* we used the 5.5 kb HindIII fragment from clone λbDm2030, described by Meyerowitz and Hogness (1982) who originally named this element *roo*. Based on their restriction map, the probe contains only a very small amount of other, flanking DNA.

As a probe for detecting DNA sequences in *D. hydei* that are similar to the binding sites for the *suppressor of Hairy-wing* [*su(Hw)*] protein of *D. melanogaster*, we used the 367 bp XmnI-BspAI DNA fragment containing nucleotide positions 675 - 1041 of the *D. melanogaster gypsy* element. This fragment specifically binds the

su(Hw) protein *in vitro* (Spana et al. 1988). The probe was provided by D.-H. Lankenau.

Probes were labelled by incorporation of [^{32}P]-dCTP by nick translation and hybridized to Southern blots of EcoRI and HindIII digested genomic DNA of *D. hydei* as described in Chapter 2. For testing whether families of transposable elements shared between *D. melanogaster* and *D. hydei* had any copies on the *D. hydei Y* chromosome, we compared the hybridization patterns on genomic DNA of males and females. Blots were initially washed at low stringency in 2 x SSC/0.1 % (w/v) SDS at room temperature (RT), followed by more stringent washing at lower salt concentrations and at higher temperatures. Stringent washes were in 0.1 x SSC at 65°C. 1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate with pH at 7.2.

In situ hybridization. Restriction fragments were subcloned in the pBluescript II KS+ plasmid vector (Stratagene). Digoxigenin (DIG)-UTP-labelled, strand-specific probes were generated by *in vitro* transcription from linearized plasmids, following protocols from Boehringer Mannheim. *In situ* hybridization on polytene chromosomes and transcript *in situ* hybridization to fixed testis tissue was performed as described in Chapter 2.

Table 1. *Gypsy* elements from the *D. hydei Y* chromosome

clone	element	position[1]	length (bp)	orientation[2]	corresponding position in *D.m. gypsy*[3]
DhNo90		221 - 5246	5026	+	1317 - 6826
DhNo86		1 - 4543	4543	+	2108 - 7021
DhNo19	1	8821 - 14490	5670	-	1413 - 7006
DhNo19	2	4021 - 6234	2214	+	4096 - 6519
DhNo19	3	2348 - 3012	665	+	4307 - 4976
DhNo19	4	1063 - 1758	696	-	3865 - 4606
DhNo19	5	646 - 982	337	-	5262 - 5661
DhNo19	6	224 - 620	397	-	6794 - 7213
MY3	1	247 - 503	257	+	1577 - 1841
MY3	2	504 - 1173	670	+	2355 - 3028
DhNocos6	1	n.d.[4]	228	n.d.	2915 - 3141
DhNocos6	2	n.d.	53	+	6954 - 7008
DhMiF8	1	808 - 1186	379	n.r.[5]	74 - 482
DhMiF8	2	1194 - 2126	923	n.r.	6378 - 7314

Notes:

[1] Numbers refer to the corresponding positions of published sequences in the case of MY3 (Vogt and Hennig 1986b; EMBL accession number X04811) and DhMiF8 (Huijser et al. 1988, EMBL accession number X13305), or of sequences that have only been deposited in the EMBL database. Accession numbers are for DhNo90: X74536, X74537 and X74543; for DhNo86: X74539, X74540, X74541 and X74542; for DhNo19: X74538; for DhNocos6: X74882, X74883, X74884, X74885 and X74886

[2] Elements with the same transcriptional orientation as the ayl repeats from the same clone are indicated by a "+" sign, those with an opposite orientation by a "-" sign

[3] Numbers refer to nucleotide positions in the *gypsy* element of *Drosophila melanogaster*, as determined by Marlor et al. (1986)

[4] Not determined

[5] Not relevant, as this is a clone from the long arm of the Y chromosome, which does not carry ayl repeats

Results

Gypsy *is a* Y-*associated sequence in* D. hydei

The present work is based on an analysis of genomic clones, shown in Figs. 1 and 2. These clones were isolated using as a probe repeats of the *Y*-specific ay1 family (Vogt and Hennig 1986b, Chapter 2). Repeats of this family of repetitive DNA sequences are specifically transcribed in the lampbrush loop pair *Nooses* (Vogt et al. 1982; Vogt and Hennig 1986a). In addition to ay1 repeats, these clones contain other, *Y*-associated DNA sequences, which are specifically transcribed in the *Nooses* as well (Chapters 2 and 5). Therefore, the clones chosen for our analysis represent potential segments of the *Nooses* loop pair.

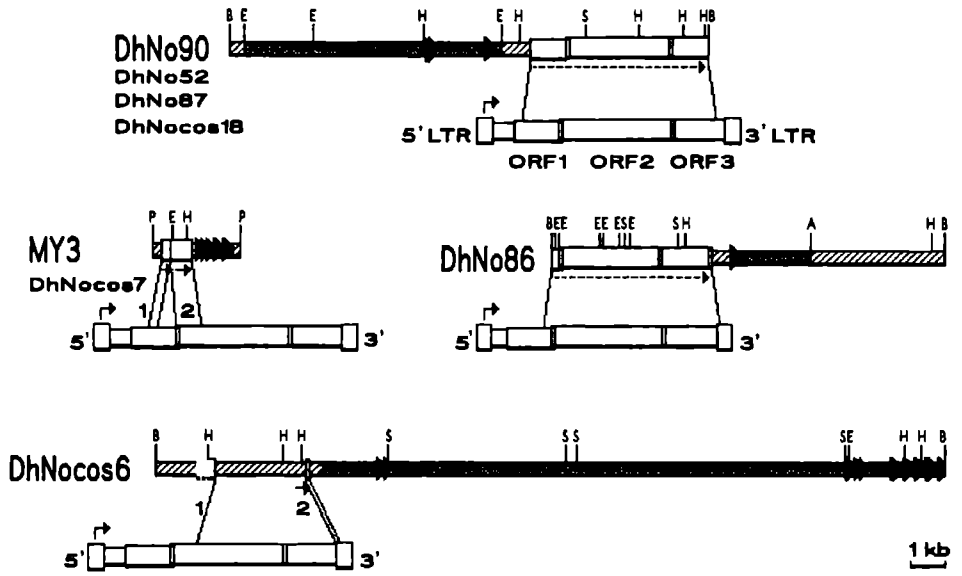


Fig. 1 Structural organization of *D hydei* genomic clones containing ay1 and *gypsy* sequences in an orientation compatible with transcription in the *Nooses* lampbrush loop pair The restriction maps shown are of the clones printed in *large letter type* Clones names in *small letter* type refer to other clones containing a similar *gypsy* element Restriction fragments harboring ay1 repeats are indicated by *dark shading* Individual ay1 repeats, as identified by sequence alignment with the original 393 bp-long ay1 repeat described by Vogt and Hennig (1986b), are represented by *dark triangles*, which point to the direction of transcription of ay1 in the *Nooses* loop pair *Gypsy* sequences are represented as *white rectangles*, with *arrows* below each sequence pointing to the direction of transcription of *gypsy* in the *Nooses* Clones MY3 and, possibly, also DhNocos6 contain more than one *gypsy* sequence, which are indicated by *numbers* that correspond to those in Table 1 and in the text The orientation of the DhNocos6-1 fragment was not determined For each *gypsy* sequence, the position of the corresponding region of the *D melanogaster gypsy* element (Marlor et al 1986) is indicated as well Restriction fragments that do not hybridize to ay1 nor to *gypsy* probes are indicted by *diagonal hatching* DhNocos6 may contain a larger *gypsy* sequence than indicated, as we have only limited DNA sequence data Abbreviations are *LTR* long terminal repeat, *ORF* open reading frame The *small arrow* above the 5' LTR indicates the transcription start of *gypsy* (Arkhipova et al 1986) Restriction enzymes are *A*, AvaI, *B*, BamHI, *E*, EcoRI, *H*, HindIII, *P*, PstI, *S*, SalI

Transcribed *Y*-associated sequences of lambda clones DhNo19, DhNo86 and DhNo90 were found to cross-hybridize with one another. By DNA sequence analysis they were all found to have a high degree of similarity to the *gypsy* retrotransposon (see Chapter 5). Altogether, 10 different *gypsy* sequences from ayl-containing clones were completely or partially sequenced (Table 1). They were named according to the clone in which they were identified and vary in size from 53 bp to 5.7 kb. DhNo86 and DhNo90 each contain one large *gypsy* sequence (Fig. 1). The plasmid clone MY3 (Vogt and Hennig 1986b) contains two, and the cosmid clone DhNocos6 at least two *gypsy* sequences (Fig. 1). DhNo19 was entirely sequenced and contains six different *gypsy* sequences (Fig. 2).

We performed nucleotide comparisons between each *Y*-associated *D. hydei gypsy* sequence and the *gypsy* elements from *D. melanogaster* (Marlor et al. 1986), *D. virilis* (Mizrokhi and Mazo 1991) and the sequence of a *gypsy* element of *D. subobscura* (Alberola and de Frutos 1993) (Table 2). High degrees of nucleotide similarity were found between *gypsy* from *D. virilis* and several of the *D. hydei* sequences, but in other cases the compared DNA sequences are diverged close to a complete randomization. However, alignments of the deduced amino acid sequences unequivocally identify the *Y*-associated sequences as *gypsy* retrotransposons (Table 3).

Gypsy *is a major constituent of the* Nooses *lampbrush loop pair*

Several lines of evidence indicate that *gypsy* represents a major fraction of the *Y*-associated DNA sequences from the *Nooses* lampbrush loop pair. From Southern blots of genomic DNA we estimated that there are about ten copies of the DhNo90 *gypsy* element and an additional 10 copies of the DhNo86 *gypsy* element on the *Y* chromosome (Chapter 2). *In situ* hybridization of the DhNo90 element on metaphase chromosomes indicates that these *gypsy* elements are clustered together on the short arm of the *Y*, at a position where also ayl sequences are located (Chapter 3). As Southern blots of genomic DNA did not reveal a tandem repetition of any of the *Y*-associated *gypsy* elements, they all must be interspersed with other

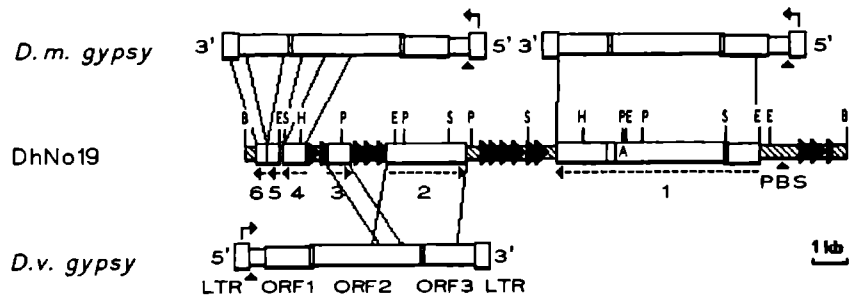


Fig. 2. Structural organization of clone DhNo19, which contains *gypsy* sequences in both orientations relative to ayl. Individual ayl repeats, *gypsy* sequences and their respective transcriptional orientations are indicated as in Fig. 1. Sequences without similarity to sequences in the nucleotide databases are represented by *diagonal hatching*. The *small triangle* indicates the position of the primer binding site (abbreviated *PBS*), as shown in more detail in Fig. 6A. The *A* between ORF2 and ORF3 of DhNo19-1 *gypsy* indicates a 36-bp poly(A)-tail (see text). Restriction enzymes and other abbreviations are as in Fig. 1

Table 2. Percentage of nucleotide identity between all *gypsy* elements[1]

	D.v.	*D.s.*	90	86	19-1	19-2	19-3	19-4	19-5	19-6	MY3-1	MY3-2	cos6-1	cos6-2	MiF8-1	MiF8-2
D.m.	73	77	64	71	60	72	74	64	67	53	59	55	62	78	67	57
D.v.		92	65	90	59	93	93	65	60	54	58	55	65	69	60	56
D.s.			66	85	64	88	88	65	-	-	-	-	-	-	-	-
90				61	57	64	64	61	64	-	53	58	62	-	-	60
86					59	85	88	65	56	51	-	53	63	74	-	60
19-1						59	58	64	43	45	61	59	69	58	-	57
19-2							95	63	56	-	-	-	-	-	-	63
19-3								61	-	-	-	-	-	-	-	-
19-4									-	-	-	-	-	-	-	-
19-5										-	-	-	-	-	-	-
19-6											-	-	-	64	53	60
MY3-1												-	-	-	-	-
MY3-2													92	-	-	-
cos6-1														-	-	-
cos6-2															64	64
MiF8-1																59

Note:
[1] A "-" sign indicates that the respective sequences do not overlap and can therefore not be aligned. Abbreviations are *D.m.*: *D. melanogaster*, *D.v.*: *D. virilis*, *D.s.*: *D. subobscura*. Other abbreviations refer to the *D. hydei Y*-associated *gypsy* elements listed in Table 1

Table 3. Percentage of amino acid identity between *gypsy* ORFs[1]

	ORF1				ORF2				ORF3			
	D.v.	90	86	19-1	*D.v.*	90	86	19-1	*D.v.*	90	86	19-1
D.m.	72	52	66	48	83	67	77	65	75	51	70	43
D.v.		50	92	44		65	89	63		49	85	41
90			46	37			60	56			47	36
86				38				59				36

Note:
[1] Abbreviations are as in Tables 1 and 2

Table 4. Percentage nucleotide identity between *gypsy* LTR sequences[1]

	D.v.	19-6	MiF8-1	MiF8-2
D.m.	66	42	67	54
D.v.		52	60	54
19-6			53	61
MiF8-1				59

Note:
[1] Abbreviations are as in Tables 1 and 2

DNA sequences, most likely with ayl repeats. However, we do not know whether all these copies are located within the 260 kb *Nooses* lampbrush loop, and small *gypsy* fragments, as those in MY3 and DhNocos6, may have escaped detection.

However, it is possible to derive a minimum value for the abundance of *gypsy* in the *Nooses* from the *gypsy* elements in our collection of ayl-containing clones. Hybridization experiments to *Nooses* transcripts, both *in situ* and on Northern blots, show that only one strand of ayl (Lifschytz and Hareven 1985; also see Chapter 4) and only the coding strand of *gypsy* are transcribed in the loop (Chapter 5). We therefore determined for each clone containing ayl and *gypsy* whether they were transcribed from the same DNA strand. One or several of the ayl repeats in each of these clones were sequenced. Consistent with the hybridization experiments to loop transcripts *in situ* and on Northern blots, all sequenced ayl repeats within a particular clone had the same orientation (Figs. 1 and 2).

As pointed out previously (Chapter 5), DhNo19 cannot be located within the *Nooses* transcription unit, since it contains *gypsy* sequences in both orientations relative to ayl (Fig. 2). All other clones may be located within the transcription unit (Fig. 1). The single *gypsy* element in DhNo86 and also that in DhNo90 are both transcribed from the same DNA strand as ayl. This is also the case for the two *gypsy* sequences in MY3. In DhNocos6 only the orientation of the 53 bp DhNocos6-2 sequence relative to ayl was determined, but its orientation is consistent with the possibility that this clone is also located within the *Nooses* transcription unit.

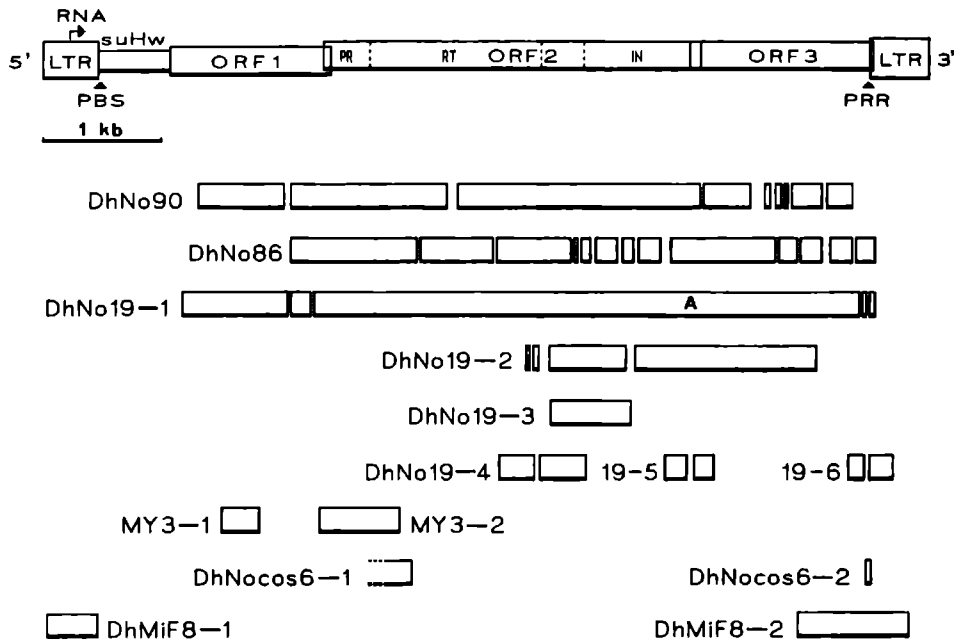


Fig. 3. Alignment of all sequenced *gypsy* fragments from the *D. hydei Y* chromosome with the *gypsy* element of *D. melanogaster* (Marlor et al. 1986). In the *D. hydei* sequences, all deletions with a size of at least 10 bp are indicated. Symbols and abbreviations are as in the previous figures, with the addition of *suHw*: region of *gypsy* containing the binding sites for the *suppressor-of-Hairy wing* protein

By cross-hybridization experiments, the DhNo90 *gypsy* element was detected in three additional ayl-containing clones (DhNocos18, DhNo52 and DhNo87). These three clones have restriction maps that are very similar to that of DhNo90 but each of them contains a unique arrangement of ayl repeats. Although we did not determine the relative orientations of ayl and *gypsy* in these three additional clones by DNA sequence analysis, the similarities of their restriction maps to that of DhNo90 indicate that ayl and *gypsy* are transcribed from the same DNA strand. At least four copies of the DhNo90 *gypsy* element may therefore, be present within the *Nooses*. By comparison of restriction maps and partial sequence analysis, we further detected an additional copy of the MY3 clone in DhNocos7 (Chapter 2).

Thus, the eight clones listed in Fig. 1 represent possible segments of the *Nooses* transcription unlt, which has a length of about 260 kb (Grond et al. 1983). Together, these clones contain about 155 kb of DNA. As determined from their restriction maps, a maximum of 100 kb consists of ayl sequences and at least 55 kb of other, *Y*-associated DNA sequences (Chapter 2). From these 55 kb, at least 27 kb are *gypsy* sequences. Thus, if these clones are representative for the organization of the DNA within the loop, two-thirds of the DNA transcribed in the *Nooses* (170-180 kb) is occupied by ayl sequences, and one-third (80-90 kb) by *Y*-associated DNA sequences. At least half of the *Y*-associated DNA seems to consist of *gypsy* sequences.

Sequence alignments reveal that Y*-associated* gypsy *elements are in a process of gradual and random degeneration*

In order to characterize the *gypsy* sequences from the *Nooses* lampbrush loop in greater detail, we aligned them with the *gypsy* element of *D. melanogaster* (Marlor et al. 1986). (Fig. 3). All sequences contain deletions, precocious stop codons and frame shifts, which all can occur at virtually any position within the element. Most deletions are small (10-20 bp). Intact open reading frames (ORFs) were not found within more than 20 kb of *Y*-associated *gypsy* DNA sequences. Further, all sequences are truncated to various extent at either the 5' or the 3' end, or at both ends.

We performed all possible pair-wise sequence comparisons between the defective *Y*-associated *gypsy* elements from *D. hydei* and the elements from *D. melanogaster*, *D. virilis* and *D. subobscura* (Table 2). On this basis, two types of *Y*-associated *gypsy* sequences could be distinguished. The DhNo86, DhNo19-2 and DhNo19-3 elements have at least 90% overall nucleotide identity with the *D. virilis* element, and at least 71% with that of *D. melanogaster*. This suggests that a functional *gypsy* element of *D. hydei* is very similar to that of *D. virilis*, and perhaps even identical. All other *D. hydei* sequences had much lower percentages of similarity (53-67%) to the elements of both other species. For comparison, the *gypsy* elements of *D. melanogaster* and *D. viliris* have 73% overall nucleotide identity.

When the *Y*-associated *D. hydei* sequences were compared among themselves (Table 2), it appeared that similarities were, in general, lower than between the *gypsy* elements of *D. melanogaster* and *D. virilis*. A high degree of similarity was detected only in two exceptional situations, first when the region of overlap was short, as for example between MY3-2 and DhNocos6-1, and second between the

sequences with high similarity to the *D. virilis* element. Thus, the *Y*-associated *gypsy* elements of *D. hydei* do not share conserved sequences. They seem to be gradually and randomly losing their sequence homology with the ancestral element that presumably is very similar to the *gypsy* element of *D. virilis*.

Gypsy elements from the lampbrush loop lack sequences required for transcription and transposition

Sequences required for transcription of *gypsy* have been identified previously. A promoter for *gypsy* transcription as well as transcription termination signals are contained within the long terminal repeat (LTR) (Arkhipova et al. 1986; Jarrell and Meselson 1991). However, due to the 5' and 3' truncation, complete LTRs were missing in the *gypsy* sequences from the *Nooses* loop.

This statement is based on the reconstruction of a putative LTR of a *D. hydei gypsy* element from sequences in the so-called "unidentified insertion" in the *micropia* retrotransposon DhMiF8 (Table 1; Fig. 4). This clone was isolated by microdissection of the lampbrush loop pairs *Threads* and *Pseudonucleolus* on the long arm of the *D. hydei Y* chromosome (Hennig et al. 1983; Huijser et al. 1988) and therefore it is of *Y* chromosomal origin. The LTRs of the *D. melanogaster* and *D. virilis gypsy* elements can be aligned only by introducing gaps, and their overall level of sequence conservation is less than that of the ORFs (Mizrokhi and Mazo 1991). Between the putative *D. hydei gypsy* LTRs from DhMiF8 and those of the other two species, overall sequence similarity is low as well (Table 4). However, the alignment of the *D. hydei* LTRs with those of the other two species requires

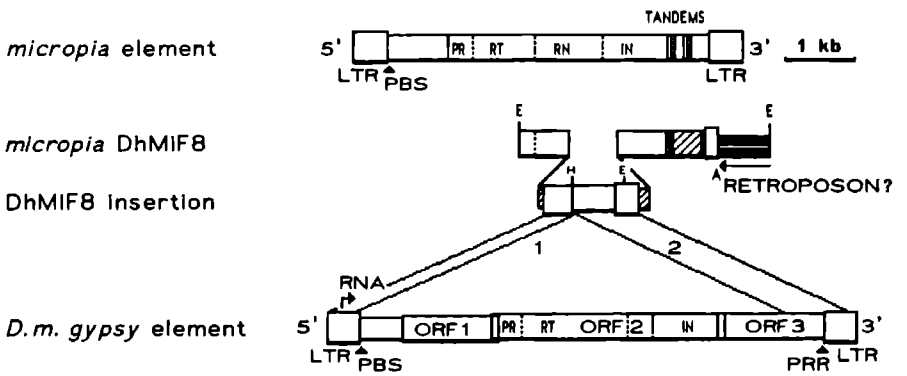


Fig. 4. Structural organization of the *Y*-associated *micropia* element DhMiF8 of *D. hydei*. DhMiF8 was originally described by Huijser et al. (1988), who identified an insertion in the *micropia* sequence that appears to consist of sequences from the 5' and 3' ends of *gypsy* (numbered 1 and 2 respectively, also see Fig. 3). Sequences in DhMiF8 without similarities to sequences in the nucleotide database are represented by diagonal hatching. The functional domains of ORF2 of *gypsy* and *micropia* are indicated as follows. *PR*: protease, *RT*: reverse transcriptase, *RN*: ribonuclease, *IN*: integrase domain. Detailed descriptions of the *micropia* element and of DhMiF8 are given by Huijser et al. (1988) and by D.-H. Lankenau et al. (1990). Restriction enzymes and other abbreviations are as in Fig. 1, with the addition of *PRR*: purine rich region (also see Fig. 6B)

several identical gaps (Fig. 5). In addition, the poly-adenylation signal as well as sequences important for termination of transcription are conserved between the *gypsy* LTRs of the three species.

Using the reconstructed, putative *D. hydei gypsy* LTR, we found that the long DhNo86 *gypsy* element is truncated in the 5' part of the 3' LTR. This was also the case for the DhNo19-1 element, which cannot, however, be located within the *Nooses* because of the opposite orientations of the *gypsy* sequences within DhNo19. Also, several of the sequences required for *gypsy* transposition were missing in the elements from putative *Nooses* segments. Immediately downstream of the 5' LTR of *gypsy* is the binding site for $tRNA_1^{lys}$, which serves as a primer for synthesis of "minus" strand DNA, the first detectable DNA product of reverse transcription of

Fig. 5 (next page). Sequence alignment of LTRs from the *gypsy* elements of *D. melanogaster*, *D. virilis* and the *Y*-associated *gypsy* elements of *D. hydei*. Sequence positions that are identical in all, or in all except one sequence, are indicated by an *asterisk* (*). Gaps introduced to increase the similarity are indicated by *dots*. The transcription initiation site of *D. melanogaster gypsy* is indicated by an *L-shaped arrow*. Nucleotides important for initiation of transcription in *D. melanogaster gypsy* (Arkhipova et al. 1986; Jarrell and Meselson 1991) are *boxed*, as well as a polyadenylation signal that is present in the sequences from all three species. The termination site of transcription in *D. melanogaster gypsy* is indicated by *vertical arrows* (Arkhipova et al. 1986). The *D. melanogaster* and *D. virilis* LTR sequences were taken from Marlor et al. (1986) and Mizrokhi and Mazo (1991), respectively. *MiF8-1* and *MiF8-2* refer to the *gypsy* LTR sequences in *micropia* DhMiF8 (Huijser et al. 1988) as shown in Fig. 4. The sequence of DhNo19 shown is reverse complementary to that containing the ay1 repeats in their transcribed orientation

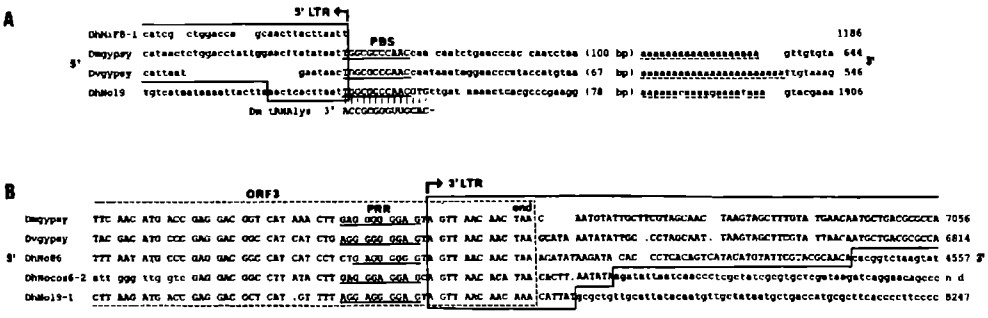
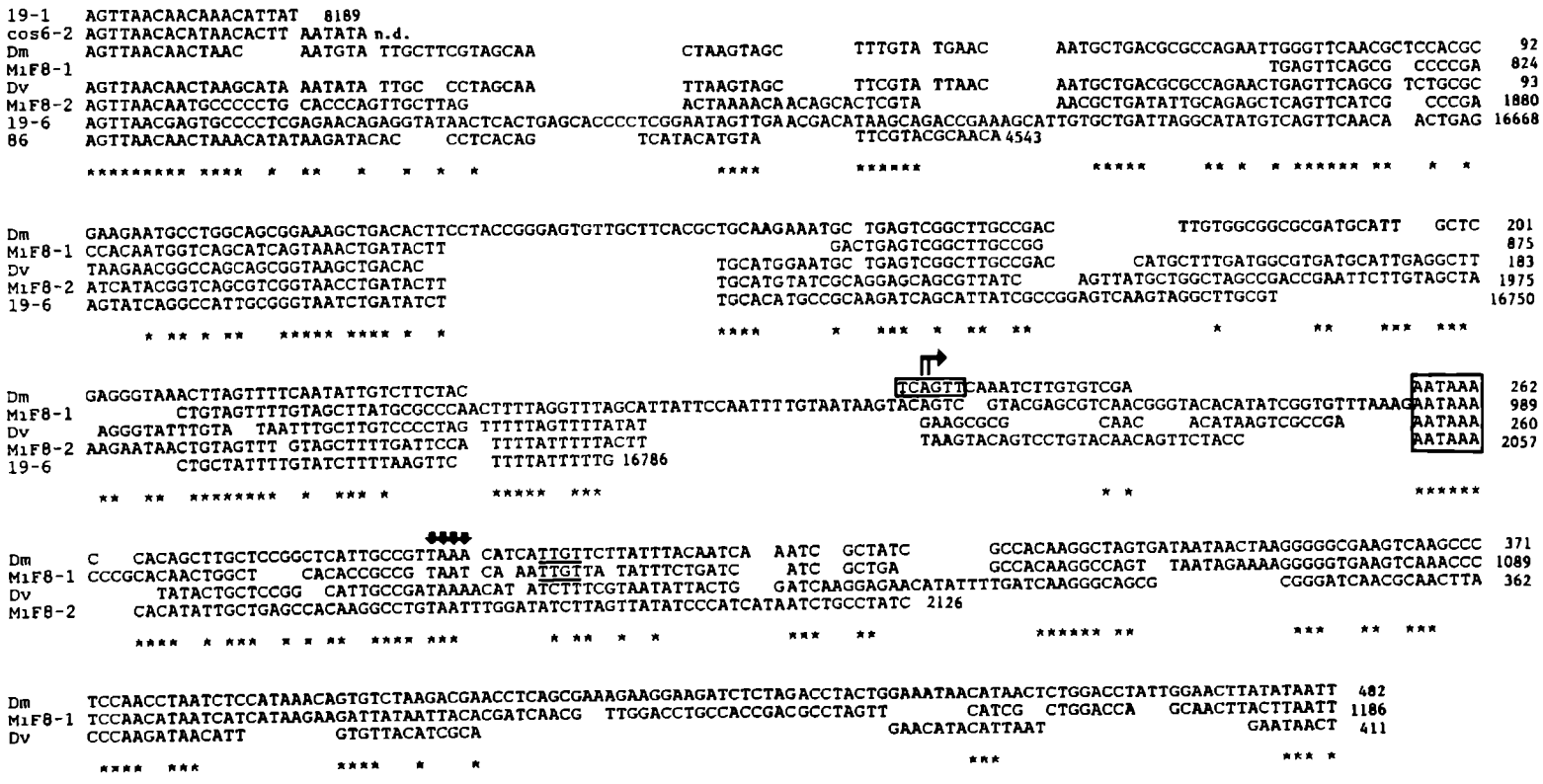


Fig. 6 A,B. Sequence alignments around the primer binding sites (**A**) and purine rich regions (**B**) from the *gypsy* elements of the three species. In **A**, the primer binding site (*PBS*) of *gypsy*, as determined by sequence alignment with *D. melanogaster* $tRNA_1^{lys}$, is *underlined*. The 3' nucleotide of the 5' LTR is the same as the 5' nucleotide of the PBS. LTR sequences are *boxed*. In all three species, there is an A-rich region 91-125 bp downstream of the PBS. In **B**, the purine rich region (*PRR*) is *underlined*. *Gypsy* sequences are in *capitals*. All three ay1-associated *D. hydei* sequences terminate in the 5' part of the 3' LTR. Gaps, which were introduced in the sequences to increase the similarity, are indicated by *dots*. The *D. melanogaster* (*Dmgypsy*), and *D. virilis* (*Dvgypsy*) sequences correspond to those described by Marlor et al. (1986) and Mizrokhi and Mazo (1991), repectively. The *D. hydei* DhMiF8 sequence was taken from Huijser et al. (1988). The sequence of DhNo19 shown is reverse complementary to the strand containing the ay1 repeats in their transcriptional orientation. n.d.: not determined


19-1 AGTTAACAACAAACATTAT 8189
cos6-2 AGTTAACACATAACACTT AATATA n.d.
Dm AGTTAACAACTAAC AATGTA TTGCTTCGTAGCAA CTAAGTAGC TTTGTA TGAAC AATGCTGACGCGCCAGAATTGGGTTCAACGCTCCACGC 92
M1F8-1 TGAGTTCAGCG CCCCGA 824
Dv AGTTAACAACTAAGCATA AATATA TTGC CCTAGCAA TTAAGTAGC TTCGTA TTAAC AATGCTGACGCGCCAGAACTGAGTTCAGCG TCTGCGC 93
M1F8-2 AGTTAACAATGCCCCCTG CACCCAGTTGCTTAG ACTAAAACA ACAGCACTCGTA AACGCTGATATTGCAGAGCTCAGTTCATCG CCCGA 1880
19-6 AGTTAACGAGTGCCCCTCGAGAACAGAGGTATAACTCACTGAGCACCCCTCGGAATAGTTGA ACGACATAAGCAGACCGAAAGCATTGTGCTGATTAGGCATATGTCAGTTCAACA ACTGAG 16668
86 AGTTAACAACTAAACATATAAGATACAC CCTCACAG TCATACATGTA TTCGTACGCAACA 4543
Dm GAAGAATGCCTGGCAGCGGAAAGCTGACACTTCCTACCGGGAGTGTTGCTTCACGCTGCAAGAAATGC TGAGTCGGCTTGCCGAC TTGTGGCGGCGCGATGCATT GCTC 201
M1F8-1 CCACAATGGTCAGCATCAGTAAACTGATACTT GACTGAGTCGGCTTGCCGG 875
Dv TAAGAACGGCCAGCAGCGGTAAGCTGACAC TGCATGGAATGC TGAGTCGGCTTGCCGAC CATGCTTTGATGGCGTGATGCATTGAGGCTT 183
M1F8-2 ATCATACGGTCAGCGTCGGTAACCTGATACTT TGCATGTATCGCAGGAGCAGCGTTATC AGTTATGCTGGCTAGCCGACCGAATTCTTGTAGCTA 1975
19-6 AGTATCAGGCCATTGCGGGTAATCTGATATCT TGCACATGCCGCAAGATCAGCATTATCGCCGGAGTCAAGTAGGCTTGCGT 16750
Dm GAGGGTAAACTTAGTTTTCAATATTGTCTTCTAC TCAGTTCAAATCTTGTGTCGA AATAAA 262
M1F8-1 CTGTAGTTTTGTAGCTTATGCGCCCAACTTTTAGGTTTAGCATTATTCCAATTTTGTAATAAGTACAGTC GTACGAGCGTCAACGGGTACACATATCGGTGTTTAAAG AATAAA 989
Dv AGGGTATTTGTA TAATTTGCTTGTCCCCTAG TTTTTAGTTTTATAT GAAGCGCG CAAC ACATAAGTCGCCGA AATAAA 260
M1F8-2 AAGAATAACTGTAGTTT GTAGCTTTTGATTCCA TTTTATTTTTACTT TAAGTACAGTCCTGTACAACAGTTCTACC AATAAA 2057
19-6 CTGCTATTTTGTATCTTTTAAGTTC TTTTATTTTTG 16786
Dm C CACAGCTTGCTCCGGCTCATTGCCGTTAAA CATCATTGTTCTTATTTACAATCA AATC GCTATC GCCACAAGGCTAGTGATAATAACTAAGGGGGCGAAGTCAAGCCC 371
M1F8-1 CCCGCACAACTGGCT CACACCGCCG TAAT CA AATTGTTA TATTTCTGATC ATC GCTGA GCCACAAGGCCAGT TAATAGAAAAGGGGGTGAAGTCAAACCC 1089
Dv TATACTGCTCCGG CATTGCCGATAAAACAT ATCTTTCGTAATATTACTG GATCAAGGAGAACATATTTTGATCAAGGGCAGCG CGGGATCAACGCAACTTA 362
M1F8-2 CACATATTGCTGAGCCACAAGGCCTGTAATTTGGATATCTTAGTTATATCCCATCATAATCTGCCTATC 2126
Dm TCCAACCTAATCTCCATAAACAGTGTCTAAGACGAACCTCAGCGAAAGAAGGAAGATCTCTAGACCTACTGGAAATAACATAACTCTGGACCTATTGGAACTTATATAATT 482
M1F8-1 TCCAACATAATCATCATAAGAAGATTATAATTACACGATCAACG TTGGACCTGCCACCGACGCCTAGTT CATCG CTGGACCA GCAACTTACTTAATT 1186
Dv CCCAAGATAACATT GTGTTACATCGCA GAACATACATTAAT GAATAACT 411

gypsy RNA (Arkhipova et al. 1986). A remnant of the primer binding site (PBS), together with the 5' end of the *gypsy* LTR (Fig. 6A), was found only in DhNo19, at the expected position relative to the DhNo19-1 *gypsy* element (Fig. 2), but, as pointed out above, it is located outside of the transcription unit. The purine-rich region (PRR), located immediately 5' of the 3' LTR was identified in the DhNo86 and DhNocos6-2 *gypsy* elements (Fig. 6B). It is used for the synthesis of the second, "plus" DNA strand, that corresponds to the coding strand of *gypsy* (Arkhipova et al. 1986).

Thus, the sequenced *gypsy* elements lack a promoter as well as sequences for termination of transcription. It seems, therefore, that the transcribed *gypsy* elements in the *Nooses* loop can neither serve as secondary initiators of loop transcription, nor can they cause a premature arrest of loop transcription.

Absence of the su(Hw) *binding sites in the* gypsy *elements from the* Nooses

We also investigated whether, in addition to complete LTRs, the binding sites for the *suppressor-of-Hairy wing* [*su(Hw)*] protein are absent as well. The *su(Hw)* gene encodes a zinc-finger protein (Parkhurst et al. 1988) that activates *gypsy* transcription (Parkhurst and Corces 1986; Mazo et al. 1989). It is present in all cells and at all stages of development (Corces and Geyer 1991). The binding sites for the *su(Hw)* protein are located between the 5' LTR and ORF1 of *gypsy* (Spana et al. 1988; also see Fig. 3). This region of *gypsy* is responsible for mediating its mutagenic effects on the expression of adjacent genes (Geyer and Corces 1992; Smith and Corces 1992; Roseman et al. 1993). Sequencing did not give evidence for the presence of the *su(Hw)* binding sites in any of the analyzed *gypsy* sequences, not even in DhNo19, where the binding sites would be expected between the primer binding site and ORF1 of the DhNo19-1 *gypsy* sequence (Fig. 2).

To further address this question we used a probe containing the *su(Hw)* binding sites of the *D. melanogaster gypsy* element. This probe hybridized equally strong to a single 3.7 kb EcoRI fragment in DNA from males and females (Fig.7A). Together with the detection of full-length 7.4 kb *gypsy* transcripts on Northern blots of RNA from the somatic parts of male adults (D.-H. Lankenau and S. Lankenau, personal communica-

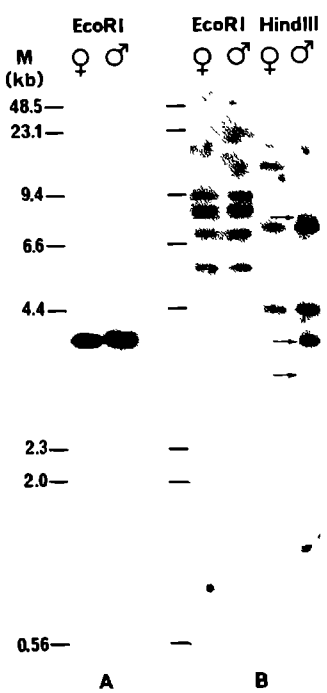


Fig. 7A, B. Southern blots of genomic DNA from *D. hydei* hybridized with a probe for the *su(Hw)* binding sites of the *D. melanogaster gypsy* element (**A**) and the *D. melanogaster* retrotransposon *17.6* (**B**). In each lane 3 µg of DNA was loaded. The probe for the *su(Hw)* binding sites hybridizes equally strong to a single EcoRI fragment in DNA from males and females, even after washing at high stringency (0.1 x SSC/65°C). The *17.6* probe hybridized only after non-stringent washing (2 x SSC/50°C). Several male-specific HindIII fragments are detected (indicated by *arrows*)

tion), this result suggests that transcribed, full-length *gypsy* elements are present in the genome of *D. hydei*, but they are not located on the *Y* chromosome. We therefore hybridized the *su(Hw)* probe to Southern blots containing restricted DNA of all ay1-containing BamHI clones. Even after washing under non-stringent conditions, no hybridization was found (not shown). Consistent with this result, there were also no signals on *Nooses* transcripts after transcript *in situ* hybridization of the *su(Hw)* probe on fixed testis tissue of *D. hydei* (not shown). These experiments strongly suggest that the region of *gypsy* containing the binding sites for the *su(Hw)* protein is absent in *gypsy* elements that are transcribed in the *Nooses*.

Multiple, independent Insertions of gypsy *Into ay1 repeats*

Our finding that the *Y*-associated *gypsy* sequences have variable degrees of similarity to a presumably ancestral, *D. virilis gypsy*-like element, suggests that the insertions of the *gypsy* sequences into the *Y* chromosome are of different evolutionary age. Thus, *gypsy* seems to have inserted into the *Y* chromosome several times independently. The analysis of clone DhNo19 (Fig. 2) fully supports this hypothesis. In the case of this clone, at least four such insertions seem to have occurred in close vicinity of each other into a previously homogeneous cluster of ay1 repeats.

DhNo19-1 corresponds to an almost full-length element and contains almost all of the protein-coding sequences of *gypsy* (only the first 334 bp of ORF1 are deleted). As a most remarkable feature, this *gypsy* element is the only one containing a poly(A)-tail. It is located immediately following the stop codon of ORF2 (see Figs. 2 and 3). The poly(A)-tail indicates that the DhNo19-1 element is the result of a retrotransposition event, which is unrelated to the normal mechanism of *gypsy* transposition, as the poly(A)-tail is in the middle, not at the 3' end of DhNo19-1.

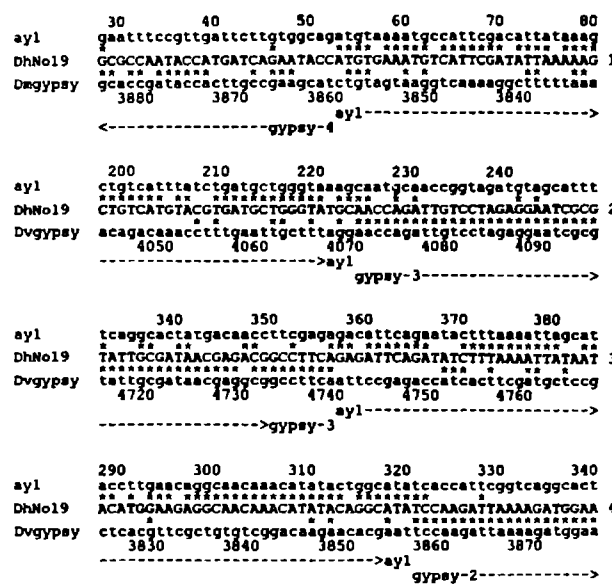


Fig. 8. Transitions between *gypsy* and ay1 sequences in DhNo19. Identical nucleotide positions are indicated by an asterisk (*). The ay1 sequence was taken from Vogt and Hennig (1986a), the *D. melanogaster gypsy* sequence from Marlor et al. (1986), and the *D. virilis gypsy* sequence from Mizrokhi and Mazo (1991). The *gypsy* sequence in the alignment at the top of the figure corresponds to the reverse complement of the coding strand of the *D. melanogaster gypsy* element

DhNo19-2 and DhNo19-3 are *gypsy* sequences with much larger terminal truncations. They must be the result of additional insertions, since they have an orientation opposite to that of all other *gypsy* sequences in DhNo19. Their much higher degree of homology to the *D. virilis gypsy* element suggests that their integration into the *Y* chromosome has occurred more recently than that of the other *gypsy* sequences in the clone. They cannot be derived from the insertion of a single element because they are separated by a cluster of ayl repeats. A remnant of a fourth insertion is represented by the three small *gypsy* sequences DhNo19-4, -5 and -6, that are not separated by ayl repeats.

Thus, the interspersion of ayl repeat clusters and *gypsy* sequences in DhNo19 resulted from multiple *gypsy* insertions into ayl repeats, which occurred in a random orientation. The transitions between several of the *gypsy* sequences in DhNo19 and their adjacent ayl sequences (Fig. 8) do not give evidence for a preferential sequence of ayl bordering *gypsy*.

Most families of transposable elements from D. melanogaster *are absent in* D. hydei

Two families of retrotransposons of *D. hydei* have members in the loop-forming male fertility genes on the *Y* chromosome: *micropia* (Huijser et al. 1988) and *gypsy* (Chapter 5). Both families are also present in *D. melanogaster* (see D.-H. Lankenau et al. 1988, 1990 for *micropia*). Therefore, other families of transposable elements of *D. melanogaster* might occur in *D. hydei* as well, and if so, they might have copies on the *Y* chromosome, possibly within the loop-forming male fertility genes.

From the literature on the transposable elements of *D. melanogaster* it appeared that the retrotransposons *412*, *297*, *copia* (Martin et al. 1983), *mdg1* (Arkhipova and Ilyin 1991), and the retroposon *jockey* (Mizrokhi and Mazo 1990) are not present in *D. hydei*. From the elements that directly transpose from DNA to DNA, the *P* element (Daniels et al. 1990a) and *hobo* (Daniels et al. 1990b) are absent.

Probes for 11 other elements of *D. melanogaster* were tested by hybridization on Southern blots of genomic DNA for their presence in *D. hydei*. Even after washing at low stringency (2 x SSC at RT) we failed to detect signals using probes for the retrotransposons *3S18*, *mdg3*, *HMS Beagle*, *springer* and *1731*, and the retroposons *I* and *doc*. From the elements that transpose directly from DNA to DNA, *pogo*, *FB4* and *BS* were found to be absent. Also the *tom* retrotransposon of *D. ananassae* was absent.

However, a probe containing sequences from the retrotransposon *17.6* (Saigo et al. 1984) was still detectable after washing in 2 x SSC at 50°C (Fig. 7B), and a probe for the retrotransposon *B104* (Meyerowitz and Hogness 1982) even after washing in 0.5 x SSC at 65°C (data not shown). Both probes did not hybridize after more stringent washing. The copy number of the *17.6*-like sequences was 5 to 10, that of the *B104*-like sequences was about 20.

By comparison of HindIII digests of DNA from females and males, it appeared that some of the copies of the *17.6*-like sequences (Fig. 7B), but not of the *B104*-like sequences, were also present on the *D. hydei Y* chromosome. Therefore, we

tested whether the *17.6* probe hybridized also to any of the ayl-containing clones, but this was not the case. When hybridized *in situ* to transcripts in fixed testis tissue, the *17.6* probe gave no signals on the *Nooses* nor on any other lampbrush loop pair. However, using the FASTA program, a sequence from the ayl-containing clone DhNo55 was found to contain 158 bp with 56% similarity to a sequence in the protease domain of ORF2 of *17.6* (see Appendix). Since no other *17.6*-like sequences were detected and the sequence similarity is low, this result may be coincidential.

In summary, from 21 families of transposable elements of *D. melanogaster* tested by us and others for their presence in *D. hydei*, only four occur in both species: *micropia*, *gypsy*, *17.6* and *B104*. Of these four families, three have members on the *Y* chromosome of *D. hydei*, but only *gypsy* is a constituent of the lampbrush loop pair *Nooses*.

Not all Drosophila *species carrying* gypsy *elements display* gypsy *transcription in a lampbrush loop pair*

Since probes for both ayl and *gypsy* hybridize only to transcripts of the *Nooses* lampbrush loop pair in *D. hydei*, and also to a "*Nooses-like* " loop pair on the *Y* chromosome of the closely related species *D. eohydei* (Chapter 5), it seems that the *Y*-associated *gypsy* sequences are transcribed only in lampbrush loops that also contain ayl repeats. We therefore investigated whether *gypsy* is transcribed in lampbrush loops of *Drosophila* species whithout ayl repeats. We used *D. repleta* and *D. virilis*, two species which both possess *gypsy* elements (Stacey et al. 1986), but lack ayl repeats (Vogt et al. 1986; Wlaschek et al. 1988).

In situ hybridization of *gypsy* probes from *D. hydei* to polytene chromosomes of female third instar larvae of *D. repleta* and *D. virilis* confirms that both species have *gypsy* sequences, which are located in the centromere-associated heterochromatin (Fig. 9A,C,E). The DhNo90 *gypsy* element hybridizes to sequences in both species, the *D. virilis*-like DhNo86 *gypsy* element only in *D. virilis*. Thus, the *D. hydei gypsy* probes are suitable for detecting *gypsy* transcripts in lampbrush loops of the two other species.

When used as probes for transcript *in situ* hybridization on testis tissue, we found however, that the DhNo86 element, but not the DhNo90 element, hybridizes to a structure in primary spermatocyte nuclei of *D. virilis* (Fig. 9B,D,F). The DhNo90 probe did not hybrize at all to testis tissue of *D. repleta*. Although the different lampbrush loops of *D. virilis* have not been described in detail, the morphology and the intranuclear location of the labelled structure in primary spermatocytes strongly indicate that it is a lampbrush loop, formed by a male fertility gene on the *Y* chromosome. The strong signal on the loop pair implies that it contains multiple copies of *gypsy*. Similar to the situation in *D. hydei* and *D. eohydei*, only the coding strand of *gypsy* was detected in the transcripts of this loop pair of *D. virilis*.

This result confirms earlier experiments of Vogt et al. (1986) who found that MY3 specifically reacts with a lampbrush loop pair in *D. virilis*, and also in *D. mulleri*. As both species have no ayl repeats, the hybridization of MY3 to the

loop transcripts is likely to be caused by the *gypsy* sequences in this clone. The phylogenetic relationships between all species where the transcription of *gypsy* in lampbrush loops has been investigated are shown in Fig. 10. It appears that several species which have *gypsy* elements in their genome lack transcription of *gypsy* in the lampbrush loops.

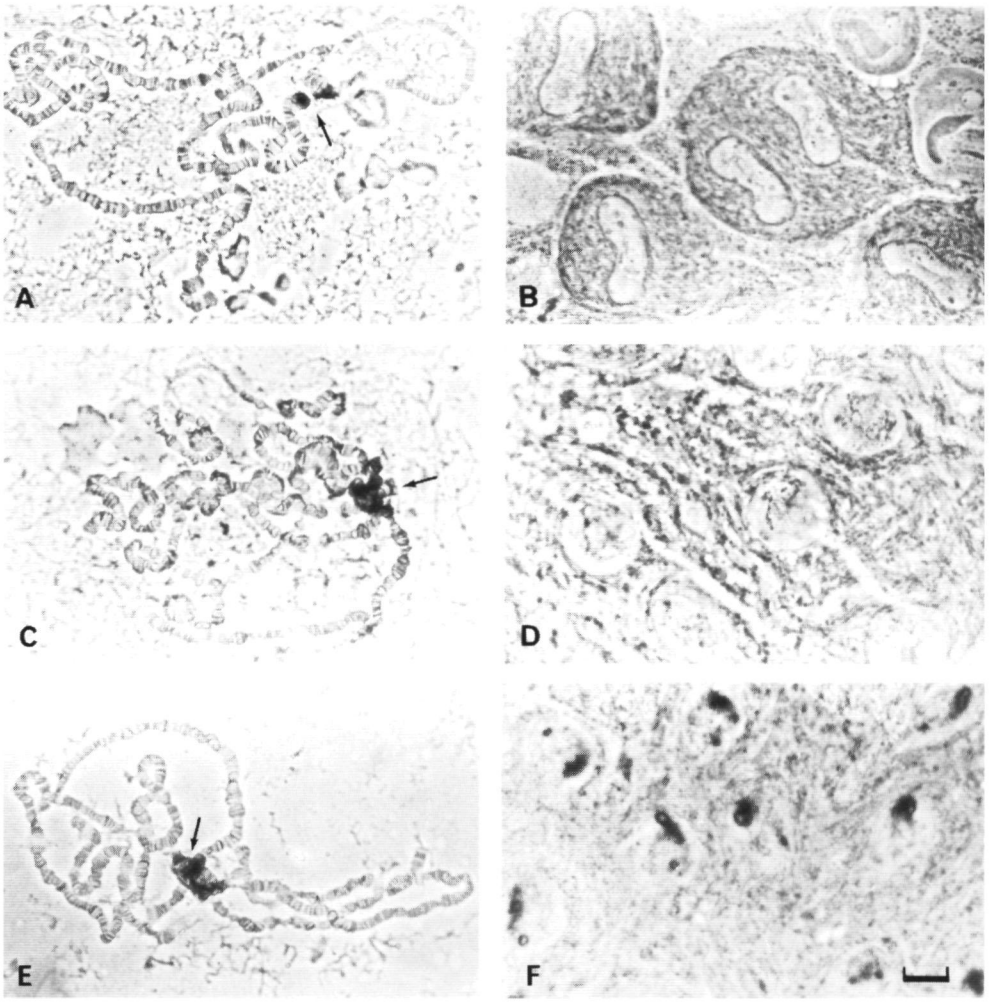
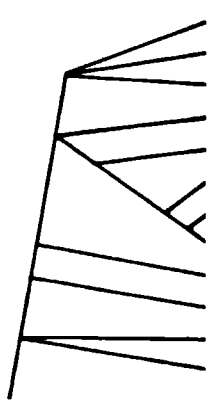


Fig. 9 A–F. Not all *Drosophila* species containing *gypsy* elements display transcription of *gypsy* in a lampbrush loop pair. *In situ* hybridization of dioxigenin-11-UTP-labelled *D. hydei gypsy* probes to salivary gland polytene chromosomes (**A**,**C**,**E**) and testis tissue (**B**,**D**,**F**) of *D. repleta* (**A** and **B**) and *D. virilis* (**C** to **F**). The DhNo90 *gypsy* element hybridizes to the centromere-associated heterochromatin in *D. repleta* (**A**) and *D. virilis* (**C**) polytene chromosomes (indicated by an *arrow*) but not to lampbrush loop transcripts in primary spermatocyte nuclei of these species (**B** and **D**, respectively). The DhNo86 *gypsy* element on the other hand, hybridizes both to the centromere-associated heterochromatin of *D. virilis* (indicated by an *arrow* in **E**), and to transcripts of a lampbrush loop pair of this species (**F**). Phase contrast. *Bar* indicates 10 μm



species	subgroup	species group	*gypsy*	ayl	loop	references
D. virilis	*virilis*	*virilis*	+	-	+	1,2,3
D. mercatorum	*mercatorum*	*repleta*	+	-	-	1,2
D. repleta	*repleta*	*repleta*	+	-	-	1,2,3
D. mulleri	*mulleri*	*repleta*	+	-	+	1,2
D. hydeoides	*hydei*	*repleta*	n.d.	-	-	2
D. eohydei	*hydei*	*repleta*	+	+	+	2,5
D. neohydei	*hydei*	*repleta*	+	+	+	2
D. hydei	*hydei*	*repleta*	+	+	+	2,4,5
D. immigrans	*immigrans*	*immigrans*	-	-	-	1,2
D. funebris	*funebris*	*funebris*	n.d.	-	-	2
D. pseudoobscura	*obscura*	*obscura*	+	-	-	1,2
D. melanogaster	*melanogaster*	*melanogaster*	+	-	-	1,2

Fig. 10. Phylogenetic distribution of the *gypsy* element and of its transcription in lampbrush loop pairs. The "cladogram" is based on the phylogenetic relationships proposed by Grimaldi (1990). The relationships within the *repleta* group are as proposed by Wasserman (1982). Columns headed "*gypsy*" and "*ayl*" indicate whether the respective sequence is present in the genome. The column headed "*loop*" indicates whether transcription of *gypsy* can be detected in lampbrush loops. References are (1): Stacey et al. (1986), who used the *gypsy* element of *D. melanogaster* as a probe; (2): Vogt et al. 1986, who used MY3 as a probe; (3): Fig. 9 of this Chapter; (4): Chapter 2, in which the DhNo86 and DhNo90 *gypsy* elements were used as a probe; (5): Chapter 5, in which the DhNo90 *gypsy* element was used as a probe. n.d.: not determined

Discussion

Characteristics of the gypsy *elements in the* Nooses *transcription unit*

Retrotransposons of the *gypsy* family are transcribed in the *Nooses* lampbrush loop pair of *D. hydei* (Chapter 5). Only the coding strand of *gypsy* is represented in transcripts of the loop. On the basis of the genomic clones in which *gypsy* is transcribed from the same strand as ayl (Fig. 1), it appears that *gypsy* occupies at least half of the *Y*-associated DNA of the *Nooses* (approximately 40-50 kb of DNA). Thus, the 27 kb of analyzed *gypsy* sequences represent at least half of all the *gypsy* elements in the loop. Therefore, it seems likely that the sample of *Y*-associated *gypsy* elements described in this paper is representative for the *gypsy* elements within the *Nooses* lampbrush loop pair.

Consequently, most, if not all, *gypsy* elements within the loop are truncated and defective. We did not detect any open reading frame which would allow the synthesis of *gypsy*-encoded proteins. In addition, the *gypsy* elements lack precisely those sequences which in *D. melanogaster* have been shown to be responsible for the mutagenic effects of *gypsy* on gene expression (reviewed by Corces and Geyer 1991), such as LTRs and the binding sites for the *su(Hw)* protein. Therefore, the *gypsy* elements in the *Nooses* loop pair appear to be functionally similar to the

gypsy elements in revertants of *gypsy*-induced mutations, in which the *su(Hw)* region is either destroyed by the insertion of another transposable element or deleted (Peifer and Bender 1988; Geyer et al. 1988).

Although we did not directly determine the sequence of each *gypsy* element that is transcribed in the *Nooses* lampbrush loop pair, we conclude that the *gypsy* elements in the *Nooses* loop, and possibly also the *Y*-associated elements outside of the loop, such as those in DhNo19, are degenerated. The defective protein coding sequences and LTR remnants of *gypsy* do not impede lampbrush loop transcription. Interestingly, the two sequenced *Y*-associated members of the *micropia* family of retrotransposons, which are constituents of the lampbrush loop pairs *Threads* and *Pseudonucleolus*, are defective as well (Huijser et al. 1988). The *micropia* element DhMiF2 has a stop codon in the reverse transcriptase domain of ORF2 and *micropia* element DhMiF8 became invaded by a *gypsy* element. In conclusion, defective retrotransposons are a general constituent of the *Y* chromosomal lampbrush loops.

A model for the inclusion of gypsy *sequences into the* Nooses *lampbrush loop*

The activity of gene *Q* during male germ cell development is associated with the unfolding of the *Nooses* lampbrush loop pair (Hackstein et al. 1982; Hackstein 1987). Since mutant alleles of male fertility genes that do not form a lampbrush loop are all sterile (Leoncini 1977; Hackstein et al. 1982; 1991), the unfolding of the loops is essential for male fertility. It now appears that a considerable fraction of the DNA transcribed in the *Nooses* loop pair consists of *gypsy* retrotransposons. Because in most cases the integration of a retrotransposon into a gene causes mutant effects on gene expression (Bingham and Zachar 1989), the question is raised how *gypsy* has become an abundant constituent of the loop-forming transcription unit without causing a sterile phenotype. This question seems to be important for understanding the function and the evolution of the lampbrush loop-forming fertility genes. By using the available data about the genomic organization of ay1 and *gypsy* (Vogt and Hennig 1986a,b; Chapters 2, 3, and 5), as well as the DNA sequence data presented in this paper, it seems possible to reconstruct some of the events that occurred during the evolutionary history of the *Nooses* transcription unit.

We postulate that the evolution of the *Nooses* loop involved the following events: *(1)* amplification of ay1 repeats into a homogeneous array, *(2)* insertion of a *gypsy* element into ay1 repeats, triggering the transcriptional activation of downstream ay1 repeats, *(3)* additional, recurrent insertions of *gypsy* into ay1 repeats, and *(4)* amplification of ay1 and *gypsy* within the transcription unit. Although we do not know the precise sequential order of these events, there are several arguments for each particular event, as discussed in greater detail below.

(1) It is most likely that repeats of the ay1 family are the original loop constituent. In all three species, *D. eohydei*, *D. neohydei*, and *D. hydei*, ay1 repeats are found exclusively on the *Y* chromosome (Vogt et al. 1986). In its basic form, the fertility gene forming the *Nooses* loop pair was present in *D. eohydei* (Chap-

ters 4 and 5) We therefore propose that the (unknown) predecessor of this species carried a tandem array of ayl repeats on the *Y* chromosome, that was not transcriptionally active, comparable to the nontranscribed array of ayl repeats on the *Y* chromosome of *D hydei* (Trapitz et al 1992, Chapters 2 and 3)

(2) The insertion of a full-length *gypsy* element into this ayl repeat array might have been the primary event that initiated the transcription of downstream located ayl repeats in the form of a lampbrush loop The promoter in the 5' LTR of *gypsy* (Jarrell and Meselson 1991) may have served as the lampbrush loop promoter The 3' LTR of *gypsy* presumably became deleted as it contains sequences that cause termination of transcription (Arkhipova et al 1986)

Although event *(2)* is highly hypothetical, there are several arguments in its support (see, however, Chapter 9, section 5 4 for an alternative view) First, the ability of retrotransposons to impose new patterns of expression on adjacent genes is well documented, especially in *Drosophila* (Bingham and Zachar 1989, McDonald 1990, Corces and Geyer 1991)

Second, *gypsy* transposes by reverse transcription of a full-length *gypsy* transcript (Arkhipova et al 1986) Elements giving rise to such transcripts exist in *D hydei*, since they are detected by hybridization of the DhNo90 *gypsy* element to Northern blots of polyadenylated RNA from the somatic tissues of flies (S Lankenau and D -H Lankenau, personal communication) However, because of their very nature as self-replicating, transposable elements, retrotransposons must also be transcribed in germ line cells Thus, the *gypsy* promoter may function during meiotic prophase of male germ cell development

Third, although heterochromatic and inactive in somatic tissues, the *Y* chromosome is decondensed during the first meiotic prophase (Kremer et al 1986), and thus, it is a potential target for invasion by transposable elements The analysis of clone DhNo19 shows that some of the *gypsy* insertions into non-transcribed ayl repeats are of a relatively recent evolutionary age Therefore, such insertions may still occur.

Fourth, only the coding strand of *gypsy* is found in transcripts of the *Nooses* loop pair of *D hydei*, just as in the *Nooses*-like loop pair of *D eohydei*, and in the loop pair of *D virilis* This is precisely what would be expected if the promoter in the 5' LTR of *gypsy* would initiate loop transcription Interestingly, also in the case of *micropia*, only the coding strand is present in the transcripts of the loop pairs *Threads* and *Pseudonucleolus* of *D hydei* (S Lankenau et al 1994)

We have no explanation for the preferential insertion of *gypsy* into ayl repeats, as all *gypsy* elements on the short arm of the *Y* chromosome are clustered together just proximal of the terminally located nucleolus organizer, at a position where also ayl repeats are located (Chapter 3) The *gypsy* element in *micropia* DhMiF8, a clone from the long arm of the *Y* chromosome (Huijser et al 1988), apparently is a rare exception Several retrotransposons integrate in specific sequences (Sandmeyer et al 1990) In *D melanogaster* the sequence 5' TA$\begin{matrix}T\\C\end{matrix}$ATA 3' has been identified as a target sequence for *gypsy* integration (Boeke and Corces 1989) This sequence occurs in about 20% of all sequenced ayl repeats (not shown). It is, however, possible that the *gypsy* element of *D hydei*, which has LTR sequences different from those of the *D melanogaster* element, also has a different

preferred integration sequence. Because of the 5' and 3' truncations of the *Y*-associated *gypsy* elements, we cannot establish a consensus sequence for the insertion of the *D. hydei* element.

(3) The variable degrees of sequence similarity of the transcribed *gypsy* elements to the *D. virilis*-like, ancestral element indicate that several times during evolution of the loop, additional inclusions of *gypsy* into the transcription unit occurred. All these elements have lost the *su(Hw)* binding sites and major parts of the LTRs. We therefore have to assume that these sequences became deleted before or during integration, since, as pointed out above, their inclusion within the loop might interfere with the continuity of loop transcription, leading to sterility.

The poly(A)-tail in the large *gypsy* element in DhNo19 Implies that this element integrated after reverse transcription of a *gypsy* mRNA. It is not impossible therefore, that several of the other insertions are the result of reverse transcription of processed transcripts that correspond to truncated forms of *gypsy*. In the case of the intracisternal A-particle (IAP), a retrotransposon family of the mouse, several variants with truncations at both the 5' and the 3' ends have been cloned as cDNAs (Kuff and Lueders 1988), indicating that truncated transcripts of the full-length IAP element exist.

Although *gypsy* can insert into ay1 repeats in both orientations, as shown by the analysis of clone DhNo19, we have to further assume that all new insertions of truncated *gypsy* elements were in the plus orientation. All repetitive DNA sequences from the loop pairs of *D. hydei* have a conserved polarity within the loop-forming transcription unit (Lifschytz and Hareven 1985; Lifschytz 1987; Trapitz et al. 1988, 1992; Chapters 2, 4 and 5). Apparently, the simultaneous presence of both strands of a certain repetitive sequence within the loop-forming transcription unit is strongly selected against.

Additional deletions of sequences of the *gypsy* elements took place after their integration into the *Y* chromosome, as documented by the many alignment gaps in the *gypsy* elements of DhNo19 (Fig. 3). The process leading to the deletion of the *gypsy* sequences also deleted adjacent ay1 sequences, because the sequence of an ay1 repeat bordering the 5' end of a *gypsy* element does not necessarily continue in the ay1 repeat at the 3' side of the element (Fig. 8).

(4) Subsequent, additional amplification events within the transcription unit generated identical or nearly identical *gypsy* sequences, as demonstrated by clones DhNo90, DhNo87, DhNo52 and DhNocos18 (also see Chapter 2). These clones contain similar, but clone-specific arangements of *gypsy* and ay1 sequences. Thus, in addition to small-scale amplifications of individual ay1 repeats (Vogt and Hennig 1986a), also amplifications on a larger scale, involving both *gypsy* and ay1, have contributed to the evolution of the *Nooses* loop. These amplification events may provide an explaination for our finding that *gypsy* accounts for about half of the *Y*-associated DNA within the *Nooses* loop, and in addition, they may also explain the conserved polarity of the transcribed repetitive DNA sequences. The nature of such mechanisms of sequence amplification within the *Y* chromosome is unclear. A possibile mechanism is unequal sister chromatid exchange between ay1 repeats. Such unequal exchanges, which occur during the mitotic, gonial divisions, have

also been proposed to explain the amplification of the ribosomal RNA genes in the male germ line of *D. melanogaster* (Endow and Atwood 1988). However, it cannot be excluded that also other mechanisms are involved.

Is the model a general model for the evolution of lampbrush loops in Drosophila*?*

The question comes up whether the structure of the *Nooses* lampbrush loop pair of *D. hydei* is a pecularity of this loop pair only. The answer to this question seems to be related to the fast rate at which the lampbrush loop-forming fertility genes are evolving, as documented by the lack of evolutionary conservation of the repetitive loop constituents (reviewed in Chapter 1). In this Chapter we have shown that in several species with *gypsy* elements in the genome *gypsy* is not a constituent of the lampbrush loops (Figs. 9 and 10). Thus, the loop-forming genes are characterized by a rapid rate of turnover of the different types of transcribed repetitive sequences.

According to our model, the lampbrush loops are initially established when the promoter in the LTR of a newly inserted retrotransposon causes the transcriptional activation of a downstream located, homogeneous array of a certain repetitive DNA sequence. Subsequently, the lampbrush loops start to evolve a more heterogeneous structure due to the accumulation of mutations in the transcribed repeats, by *de novo* insertions of retrotransposons into the transcription unit, and by sequence amplification. The model therefore predicts that loops of a recent evolutionary origin are more homogeneous than more ancient loops. On the DNA sequence level, this means that the transcribed repeats of young loop pairs are better conserved and, also, that they are less frequently interspersed with retrotransposons than those of older loops.

All available data on the DNA sequence composition of the lampbrush loop pairs of *D. melanogaster* and *D. hydei* indeed show that loops with a high degree of conservation of transcribed repeats have a low content of retrotransposons. The three lampbrush loop pairs of *D. melanogaster* consist mainly of homogeneous pentameric satellite repeat sequences (Bonaccorsi et al. 1990; Bonaccorsi and Lohe 1991; Gatti and Pimpinelli 1992). Using different retrotransposons of *D. melanogaster* as probes for transcript *in situ* hybridization, we were unable to detect their transcription in any of the lampbrush loop pairs of this species (not shown).

The loop pair *Threads*, formed by fertility gene *A* of *D. hydei* (Hackstein et al. 1982; 1991) contains long repeat arrays of the YLII and *rally* families of repetitive DNA sequences (Huijser and Hennig 1987; Trapitz et al. 1988;1992). Transcribed members of the YLII family share a higher degree of sequence similarity than those of the ay1 family (Papenbrock 1991; also see Chapter 6). Furthermore, as judged from Northern blots and from transcript *in situ* hybridization experiments (Huijser et al. 1988), the fraction of *Threads* DNA occupied by *micropia* is considerably smaller than the fraction of *Nooses* DNA occupied by *gypsy*. All these observations suggest that the *Nooses* loop pair is of a more ancient origin than the *Threads* loop pair, consistent with the cytogenetic studes of I. Hennig (1978), as discussed in Chapter 6.

Does gypsy *contribute to the function of male fertility gene* Q *?*

Evolution has apparently favoured the inclusion of multiple copies of *gypsy* in the *Nooses* lampbrush loop pair of *D. hydei*. We therefore have to discuss whether these *gypsy* elements are merely tolerated, or whether they provide essential contributions to the single, mutable function of male fertility gene *Q*.

Two lines of evidence indicate that any contribution of *gypsy* to the function of gene *Q* cannot be based on a specific DNA sequence. First, as described in this Chapter, the transcribed *gypsy* elements lack a conserved DNA sequence. They are randomly affected by point mutations and deletions, and are slowly, but gradually losing their similarity to the ancestral, *D. virilis*-like *gypsy* element. Second, if the transcription of *gypsy* in a lampbrush loop is essential for male fertility, one would expect its conservation among the different *Drosophila* species. The *gypsy* family is present in all but two of the 34 species investigated by Stacey et al. (1986), but in several of these species *gypsy* sequences could not be detected in lampbrush loop transcripts (Figs. 9 and 10). It could be argued that in species without loop transcription of *gypsy*, its function is taken over by other families of retrotransposons. But then, such a function cannot be based on a specific, conserved DNA sequence.

Following earlier indications that the loop-forming gene *kl-5* on the *Y* chromosome of *D. melanogaster* encodes a dynein protein of the sperm axoneme (Hardy et al. 1981; Goldstein et al. 1982), it has recently been shown that the region containing this gene also contains the coding sequences of a dynein β-heavy chain isoform (Gepner and Hays 1993). However, it has not been shown that the exons encoding dynein are actually located within the lampbrush loop formed by *kl-5* (see the discussion by Hennig 1993 and that in Chapter 9). We have no direct evidence that also gene *Q* is a protein coding gene. But irrespective of whether this is the case, we conclude that the characteristics of the *gypsy* elements found in the *Nooses* loop pair are most easily explained by assuming that these *gypsy* elements are co-transcribed with other, functionally important constituents of the gene. This conclusion is supported by observations described in the following Chapter: the transcription of ayl and *gypsy* does not seem to be detectably affected in at least one sterile allele of gene *Q*.

Acknowledgements. We are indebted to the various *Drosophila* workers who generously provided us with probes for transposable elements, and to Dr. Tom Hays, Dr. Dirk-Henner Lankenau, and to Susanne Lankenau for communicating unpublished data. We are also grateful to Dr. Dirk-Henner Lankenau, and to our colleagues Anna Akhmanova, Koos Miedema and Xiaoping Sun for their valuable comments on earlier versions of the manuscript. Wieli Janssen supported us with expert technical assistance.

CHAPTER 8

Transcription of repetitive DNA sequences in the lampbrush loop pair *Nooses* formed by sterile alleles of fertility gene *Q* on the *Y* chromosome of *Drosophila hydei*

Submitted for publication

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Abstract. The lampbrush loop-forming male fertility genes on the *Y* chromosome of *Drosophila* consist mainly of repetitive DNA sequences that do not code for proteins. We investigated whether the transcription of these sequences is affected in sterile alleles of male fertility gene *Q*, that forms the loop pair *Nooses*. This loop consists for approximately two-thirds of repeats of the *Y*-specific ay1 family of repetitive DNA sequences. From the remaining one-third, at least one half is represented by defective retrotransposons of the *gypsy* family. Both sequence types are interspersed throughout the loop. Using both ay1 and *gypsy* sequences as probes for transcript *in situ* hybridization, we show that, at the level of the light microscope, transcription of neither sequence is detectably affected in the loops formed by at least one male-sterile allele of gene *Q*. On Northern blots of RNA from males carrying sterile alleles, transcripts, normal in amount and size distribution, are seen. We conclude that the function of fertility gene *Q* depends on the transcription of DNA sequences in addition to ay1 and *gypsy*.

Introduction

The *Y* chromosome of *Drosophila melanogaster* is not required for male viability. It does carry however, a small number of genes essential for male fertility (Bridges 1916). Four fertility genes have been mapped on the long arm of the *Y*, and two on the short arm. In *D. hydei*, there are at least six genes on the long arm and one is located on the short arm (reviewed by Hackstein 1987; Gatti and Pimpinelli 1992). In both species, several of these fertility genes form lampbrush loop pairs during the meiotic prophase of male germ cell development (Meyer et al. 1961; Meyer 1963; for reviews see Hess and Meyer 1968; Hennig 1985; 1987). Deletions or mutations of these genes cause defects during advanced stages of spermatid differentiation (Hess and Meyer 1968; Hackstein et al. 1982, 1991).

Classic genetic studies indicate that in both species, all *Y* chromosomal fertility genes correspond to one complementation group. Therefore, each gene performs a single, unique function that is indispensable for fertility (reviewed by Hackstein 1987). As shown by several investigators, this function is mutable to sterility at frequencies that are about two orders of magnitude higher compared to genes on the *X*, irrespective of whether the mutations are induced by X-rays (Brosseau 1960; Hackstein et al. 1982; Hazelrigg et al. 1982), γ-rays (Kennison 1983), or EMS (Williamson 1970; Leoncini 1977). Even if P-elements are used as the mutagenic agent, the difference is approximately tenfold (U. Schäfer and Nahmias 1985).

The high susceptibility to mutations has been attributed to the exceptionally large size of the fertility genes. The distributions of sterilizing breakpoints on the *Y* chromosome of *D. melanogaster* suggest that the loop-forming genes extend over several megabases of DNA (Gatti and Pimpinelli 1983; Bonaccorsi et al. 1988). Miller spreading experiments indicate transcript sizes of 260 kb to more than 1500 kb for the lampbrush loops of *D. hydei* (Glätzer and Meyer 1981; Grond et al. 1983; de Loos et al. 1984)

However, the high mutation rate is not as easily reconciled with the DNA sequence content of the loop-forming genes. As shown by successive investigations of our laboratory (reviewed by Hennig et al. 1989; Hennig 1990), the transcription units forming the lampbrush loops of *D. hydei* contain two distinct, repetitive, DNA sequence constituents. The first type is *Y*-specific and is represented by complex and heterogeneous families of satellite-like DNA sequences without protein-coding potential (Vogt et al. 1982; Vogt and Hennig 1983,1986a; Huijser and Hennig 1987, Huijser et al. 1987, 1990). Sequences of this type were also identified by other investigators (Lifschytz et al. 1983; Wlaschek et al. 1988). The second type is called *Y*-associated and is represented by defective retrotransposons with additional copies on other chromosomes (Vogt and Hennig 1986b; Huijser et al. 1988; Chapters 2, 5 and 7). The only constant feature shared between the individual members of each repetitive sequence family is their conserved polarity within the loops (Lifschytz and Hareven 1985; Trapitz et al. 1988, 1992; Chapters 2, 4 and 5). However, individual members do not share a conserved DNA sequence and also differ greatly in length. Therefore, it is not obvious why the loop-forming genes are so extremely sensitive to all kinds of mutagens.

In this paper we investigate whether the transcription of these two types of loop constituents is affected in male-sterile alleles of fertility gene *Q*, forming the loop pair *Nooses*. Each loop is a single transcription unit with a size of 260 kb (Grond et al. 1983). Within the loop, repeats of the *Y*-specific ayl family, with a basic repeat length of 393 bp (Vogt and Hennig 1986a), are interspersed with degenerating *gypsy* retrotransposons of variable size and without protein coding potential (Vogt and Hennig 1986b; Chapter 5). Based on restriction maps of putative loop segments, we have estimated that the ayl repeats represent about two-thirds of the DNA in the loop , and that *gypsy* may account for at least half of the remaining DNA (Chapters 2, 5, and 7).

We show that both these major repetitive loop constituents, ayl and *gypsy*, can be transcribed in loops formed by a mutant allele of gene *Q*. Transcripts containing ayl repeats are present in normal amounts and size distributions on Northern blots of RNA from males carrying sterile alleles. Thus, the transcription of these repetitive DNA sequences in the *Nooses* loop pair is not detectably affected by sterile lesions in gene *Q*.

Materials and Methods

Drosophila strains. All fly strains were from our laboratory collection. Flies were kept at 18°C or 24°C on a medium containing dried yeast, cornmeal, soy flour, malt, and sugar-beet syrup, that was inoculated with live baker's yeast.

The genotypes of male flies carrying fertile alleles, sterile alleles, or deficiencies of male fertility gene *Q* are listed in Table 1. For details, see Hackstein et al. (1982) and Hackstein and Hennig (1982).

Sterile alleles. We used four different male-sterile alleles. The *ms(Y)Q2* and *ms(Y)Q4*^{ts} alleles were induced by ethylmethane sulphonate (EMS). Males carrying either one of these alleles have a *Y* chromosome of normal length in metaphase preparations from larval brains (not shown). The temperature-sensitive allele *ms(Y)Q4*^{ts} was induced by O. Leoncini in 1976. It was originally named *TS507*, but renamed by Hackstein et al. (1982). Sterile *X/ms(Y)Q4*^{ts} males were obtained either by allowing

embryos to complete development at 26°C or by transferring newly emerged males from 18°C to 26°C for 9–20 days For temperature-sensitive alleles of other fertility genes on the *Y* chromosome a temperature shift of 9 days is sufficient to cause sterility (Leoncini 1977) Sterility of *X/ms(Y)Q4*^{ts} males used for RNA isolation or for the preparation of slides for *in situ* hybridization was confirmed by mating of at least 10 sibling males of the same phenotype to virgin wild-type females, and by dissecting at least three of such males to check the absence of motile sperm

The *Df(YL)50-3 Y* chromosome was obtained following X-ray treatment of *Df(YL)50*, a *Y* chromosome with a deletion of almost the entire long arm *Df(YL)50-3* carries a wild-type allele of gene *P*, forming the loop pair *Clubs*, and a sterile allele of gene *Q* forming a *Nooses* loop pair of abnormal morphology (J H P Hackstein, personal communication)

T(X Y)56 was obtained in 1976 by X-ray treatment of a wild-type *Y* chromosome It was originally interpreted as a translocation of the long arm of the *Y* chromosome and the euchromatic arm of the *X* chromosome that did not form a visible *Nooses* loop pair However, Lifschytz and Hareven (1985) showed that ayl sequences were still present on *T(X Y)56* and also that it forms a *Nooses* loop pair that is more diffuse than the wild type loop pair

Deficiencies As negative controls to study the transcription of DNA sequences in the *Nooses*, we used males carrying a deletion of the short arm of the *Y* chromosome, and therefore missing gene *Q* and the associated loop pair *Nooses* These males either had the genotype *X/ms(Y)Q1* or *X/Df(YS)Q1* *X/ms(Y)Q1* males were obtained from crossing virgin wild-type females to *T(X,Y)59/ms(Y)Q1* males, location of the short arm of the *Y* chromosome to the *X* chromosome After its induction in 1979, *ms(Y)Q1* originally had a normal appearance in metaphase chromosome preparations, forming a lampbrush loop pair *Nooses* of apparently normal morphology However, during maintenance of the stock the short arm of the *Y* chromosome was lost (J H P Hackstein, personal communication) Absence of

Table 1. Genotypes of male flies used in this study

genotype	source	induction	loop cytology[1]	fertility genes[2]
T(X,Y)58/0	wild-type *Y*, Tubingen	X-ray	$Th^-Ps^-Tr^-Cl^-Ns^+$	*Q*
X/Df(YL)51	*Y*, w^mCo, Leiden	X-ray	$Th^-Ps^-Tr^-Cl^-Ns^+$	*Q*
X/Df(YL)50	*Y*, w^mCo, Leiden	X-ray	$Th^-Ps^-Tr^-Cl^+Ns^+$	*O, P, Q*
X/Df(YL)50-3	*Df(YL)50*	X-ray	$Th^-Ps^-Tr^-Cl^+Ns^m$	*O, P*
X/ms(Y)Q2	wild-type *Y*, Tubingen	EMS	$Th^+Ps^+Tr^+Cl^+Ns^+$	*A* to *P*
X/ms(Y)Q4^{ts}	wild-type *Y*, Tubingen	EMS	$Th^+Ps^+Tr^+Cl^+Ns^+$	*A* to *P*[3]
T(X,Y)56/0	wild-type *Y*, Geneva	X-ray	$Th^+Ps^+Tr^+Cl^+Ns^m$	*A* to *P*
X/ms(Y)Q1	wild-type *Y*, Tubingen	EMS	$Th^+Ps^+Tr^+Cl^+Ns^-$	*A* to *P*
X/Df(YS)Q1	*Y*, w^mCo, Leiden	transposition[4]	$Th^+Ps^+Tr^+Cl^+Ns^-$	*A* to *P*

Notes

[1] Primary spermatocyte cytology in squash preparations as seen under phase contrast + indicates that the corresponding loop pair displays a wild-type morphology, m that it has a modified morphology, and - that the loop pair is absent

[2] Genes *A* to *P* are on the long arm of the *Y* chromosome, gene *Q* is on the short arm For the definition and localization of the fertility genes, see Bonaccorsi et al 1981, Hackstein et al 1982, Hennig 1985, Hackstein 1987, Chapter 4

[3] These males were raised at 26°C (see Materials and Methods)

[4] Caused by spontaneous transposition of the giant w^mCo transposable element (Hackstein et al 1987)

the short arm was confirmed by inspection of brain metaphase preparations of *X/ms(Y)Q1* larvae and by lack of hybridization of ay1 probes to Southern blots of genomic DNA prepared from such males (not shown).

The *Df(YS)Q1 Y* chromosome resulted from the spontaneous transposition of the giant $w^{m}Co$ transposon into the short arm of the *Y* chromosome (J.H.P. Hackstein, personal communication). The transposon contains polytene chromosome bands 16B2-17B1 (for more details, see Hackstein et al. 1987).

Fertile alleles. As positive controls in the hybrization experiments on Northern blots of RNA we used males carrying only the short arm of the *Y* chromosome (Table 1). These males had the genotypes *T(X;Y)58/0* (Hess 1965b) or *X/Df(YL)51* (Hackstein et al. 1982), and carried the wild-type allele of gene *Q*, with all other *Y* chromosomal fertility genes lacking. We also used *X/Df(YL)50* males carrying wild-type alleles of fertility genes *O*, *P*, and *Q*.

For details of the respective crosses required for obtaining the males of the different genotypes, consult Hackstein et al. (1982).

Nucleic acid probes. For the detection of ay1 repeats we either used clone PY9 (Vogt et al. 1982; Vogt and Hennig 1983), or an individual ay1 repeat as defined by Vogt and Hennig (1986a). PY9 is a genomic PstI DNA fragment of 9 kb cloned in pBR322. The insert contains about 20 different repeats of the ay1 family. However, on Southern blots of genomic DNA from males, both PY9 and the basic ay1 repeat with a length of 393 bp give hybridization patterns that are indistinguishable (Vogt and Hennig 1986a). Therefore, the sequence complexity of the ay1 family is represented by both the smaller and larger DNA fragment.

For the *in situ* detection of *gypsy* sequences, we used a 5.8 kb BamHI-EcoRI fragment from the genomic ay1-containing clone DhNo90 (Chapter 2), that contains a 5.0 kb *gypsy* fragment of which the complete sequence has been determined (Chapters 5 and 7). It consists only of protein coding sequences of *gypsy* and has about 65% sequence identity to the corresponding sequences in the *gypsy* elements of *D. melanogaster* and *D. virilis*. Both *in situ* and on Northern blots, it specifically hybridizes to *Nooses* transcripts. This particular fragment of DhNo90 was chosen as a probe for the detection of *gypsy* sequences for two reasons. First, within DhNo90, the orientation of *gypsy* relative to ay1 is consistent with the presence of the clone within the *Nooses* transcription unit (Chapter 5). Second, the 5.8 kb fragment is also present in at least three other genomic clones. In each of these clones, it has the same orientation relative to ay1 as in DhNo90 (Chapters 2, 5 and 7). Therefore, this particular *gypsy* fragment may occur multiple times within the *Nooses* transcription unit.

Labelling of probes. For hybridization to Northern blots, purified PY9 insert DNA was labelled by nick translation using [^{32}P]-dCTP (Sambrook et al. 1989). [^{3}H]-labelled cRNA PY9 probes for radioactive *in situ* hybridization were produced as described by Vogt et al. (1982). To generate probes for nonradioactive *in situ* hybridization, we subcloned the 393 bp ay1 fragment and the 5.8 kb BamHI-EcoRI fragment from DhNo90 in pBluescript II KS+ (Stratagene). Digoxigenin-11-UTP-labelled strand-specific RNA probes were produced from linearized plasmid DNA by *in vitro* transcription using either T3 or T7 polymerase (Stratagene) following Boehringer Mannheim protocols.

Northern blots. Total RNA was isolated from whole adult males following the method of Chirgwin et al. (1979), and loaded on a denaturing agarose gel and transferred to Gene Screen Plus membranes (New England Nuclear) as described in detail by Brand and Hennig (1989). Each lane contained about 20 µg RNA. Hybridization was in 0.5 M Na_2HPO_4 (pH 7.2), 1% (w/v) bovine serum albumin, 1 mM EDTA and 7% (w/v) SDS at 65°C. Posthybridization washes were in 0.3 M Na_2HPO_4, 1% (w/v) SDS at 60°C. Since the *Y* chromosome is not required for male viability (Bridges 1916) and is

transcriptionally active only in cells of the male germ line (Hennig 1967), it can be safely assumed that in this experiment all transcripts that are detected using *Y*-specific sequences, such as ay1, as a probe, are indeed of *Y* chromosomal origin.

Transcript in situ hybridization. Radioactive transcript *in situ* hybridization was as described by Vogt et al. (1982). For the detection of lampbrush loop transcripts by nonradioactive *in situ* hybridization, we used a modification of the protocol of Tautz and Pfeifle (1989), as described in Chapter 2.

Results

Morphological appearance of the Nooses *lampbrush loop pair in wild-type males*

Fig. 1. shows the morphological appearance of the *Nooses* loop pair formed by the wild-type allele of fertility gene *Q*. In wild-type males, the loop pair is poorly visible because of the presence of the four larger loop pairs on the long arm of the *Y* chromosome (Hess 1965b). Therefore, the primary spermatocyte nucleus shown in Fig. 1A is from a *T(X;Y)58/0* male, with only the *Nooses* present. Nonradioactive transcript *in situ* hybridization of digoxigenin-labelled, loop-specific ay1 repeat probes allows a clear visualization of the *Nooses* also in primary spermatocytes of wild-type males. In favourable preparations such as that shown in Fig. 1B, the entire loop pair can be followed throughout the nucleus. Each loop is a single transcription unit of approximately 50 μm length, displaying a gradient of growing

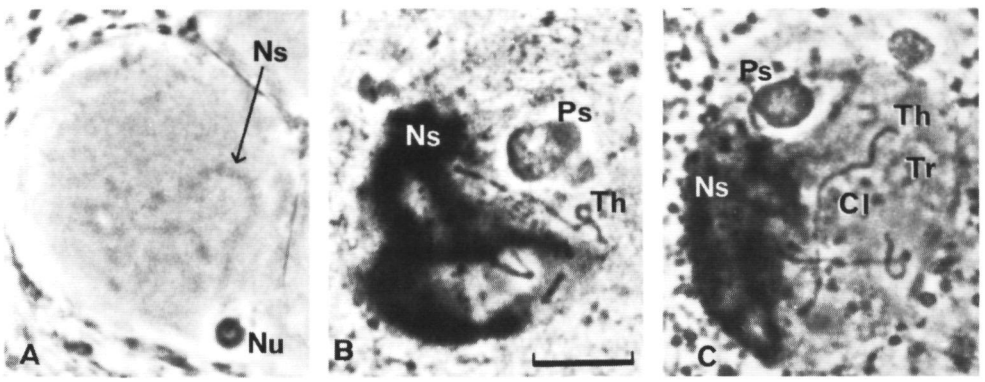

Fig. 1, A – C. Morphology of the lampbrush loop pair *Nooses*. **A** Primary spermatocyte nucleus of a male of the genotype *T(X;Y)58/0*, carrying a wild-type allele of gene *Q* and lacking all other *Y* chromosomal fertility genes. Only a morphologically normal *Nooses* lampbrush loop pair (*Ns*) is seen (indicated by an *arrow*), which unfolds from a site close to the nucleolus organizer (*Nu*). Males of this genotype were used for visualization of transcribed chromatin of the *Nooses* by the Miller spreading procedure (Grond et al. 1983; also see Fig. 3 of Chapter 1). **B** Primary spermatocyte nucleus of a wild-type male following nonradioactive transcript *in situ* hybridization of the digoxigenin-11-UTP-labelled ay1 RNA probe. Only the *Nooses* loop pair (*Ns*) is labelled. Other visible lampbrush loop pairs are *Threads* (*Th*) and *Pseudonucleolus* (*Ps*), the *Clubs* and *Tubular ribbons* are not visible. **C** Same as in **B**, but now using the DhNo90 *gypsy* probe. Again, only the *Nooses* loop pair (*Ns*) is labelled. In this nucleus, also the *Clubs* (*Cl*) and the *Tubular ribbons* (*Tr*) can be seen. Phase contrast. *Bar* represents 10 μm

loop transcripts. The distribution of the coloured precipitate indicates that the transcripts are tightly associated with the DNA axis of the loop, and also that they are extensively folded; since their length is much smaller than the length of the corresponding segment of the transcription unit. Identical results are obtained using *gypsy* sequences as a probe (Fig. 1C). In fact, when both probes are hybridized simultaneously, the two labelling patterns on the *Nooses*, detected using different fluorochromes, are very similar (see Chapter 5).

In Chapter 1, the appearance of the *Nooses* loop pair of wild-type males, as revealed in the light microscope by transcript *in situ* hybridization, has been compared to its appearance in the electron microscope, as revealed by the Miller spreading technique, using *T(X;Y)58/0* males (Grond et al. 1983). By both procedures, loop size and loop morphology are very similar (see Fig. 3 of Chapter 1). Thus, transcript *in situ* hybridization faithfully reveals the morphological features of the *Nooses* loop in wild-type males, as they were earlier observed in the electron microscope.

Male-sterile alleles of fertility gene Q *form a* Nooses *lampbrush loop pair of abnormal or of normal cytological appearance*

Previous cytogenetic analyses have shown that the loops formed by male-sterile alleles of *Y* chromosomal fertility genes are of three categories (Leoncini 1977; Hackstein et al. 1982, 1991). The loops are either absent, modified in morphology, or normal (*i.e.* indistinguishable from the wild-type at the level of the light microscope). This classification with respect to loop morphology is also possible for male-sterile alleles of gene *Q*. For the present work, we studied an allele forming a modified loop pair, and an allele forming an apparently normal loop pair.

Males of the genotype *X/Df(YL)50-3*, have a *Nooses* lampbrush loop pair of modified morphology. In primary spermatocytes two small, globular structures are visible in close proximity to the nucleolus (Fig. 2A). Therefore, they are at the same position as the *Nooses* loop pair in primary spermatocytes of wild-type

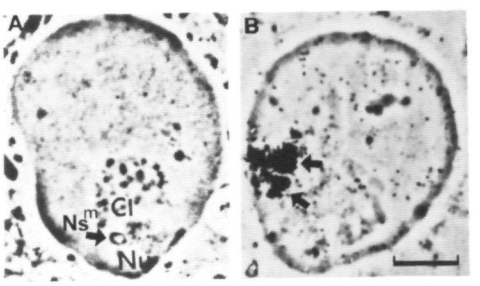

Fig. 2, A, B. Transcription of ay1 repeats in a *Nooses* loop pair of abnormal morphology formed by a sterile allele of gene *Q*. **A** Primary spermatocyte nucleus of a male of the genotype *X/Df(YL)50-3*. The *arrow* indicates the compact, round *Nooses* loop pair (*Ns*). The *Df(YL)50-3* chromosome also carries a wild type allele of fertility gene *O*, forming a normally looking *Clubs* lampbrush loop pair (*Cl*). Both loop pairs are located close to the nucleolus organizer (*Nu*). **B** Transcript *in situ* hybridization of [^{3}H]-labelled PY9 cRNA to testis tissue of an *X/Df(YL)50-3* male. The dense accumulation of silver grains on the compact *Nooses* loop pair (*arrows*) indicates that ay1 repeats are transcribed. Note that this is a different nucleus than the one shown in **A**. Phase contrast. *Bar* represents 10 μm

males (Fig. 1). Transcript *in situ* hybridization with the *Nooses*-specific probe PY9, containing only ay1 sequences, results in labelling of the two small structures (Fig. 2, B). This proves that the small, globular structures correspond to a drastically modified *Nooses* loop pair formed by the sterile allele of gene *Q* on the *Df(YL)50-3 Y* chromosome.

Preliminary ultrastructural studies of this modified *Nooses* loop pair indicate that the size of the transcription unit is less than half the size of the completely unfolded loop pair formed by the wild-type allele. In addition, the individual transcripts seem to be more closely spaced along the DNA axis of the loop than in the wild-type (R. Suijkerbuijk and W. Hennig, unpublished observations). Both these features of the *Df(YL)50-3 Nooses* loop pair may contribute to its more compact appearance in the light microscope. Thus, transcript *in situ* hybridization permits the detection of major changes in the length of the *Nooses* transcription unit.

We also studied the temperature-sensitive allele $ms(Y)Q4^{ts}$ which leads to male fertility at 18°C, but causes sterility at 26°C. At the permissive temperature, it forms a *Nooses* loop pair of normal cytological appearance, as visualized using either the ay1 or the *gypsy* probe (Fig. 3A,B). Also at the restrictive temperature, the morphology of the *Nooses* loop pair is normal and not detectably different from that at the permissive temperature (Fig. 3C,D), indicating that there are no gross alterations in loop length nor in the initiation rate of loop transcription.

We therefore conclude that an allele of gene *Q* in which the normal function of the fertility gene is destroyed, is nevertheless able to form a normally looking lampbrush loop pair. At the level of detection by cytological methods, both major types of repetitive sequence constituents, ay1 and *gypsy*, are transcribed in a pattern not detectably differerent from a wild-type situation.

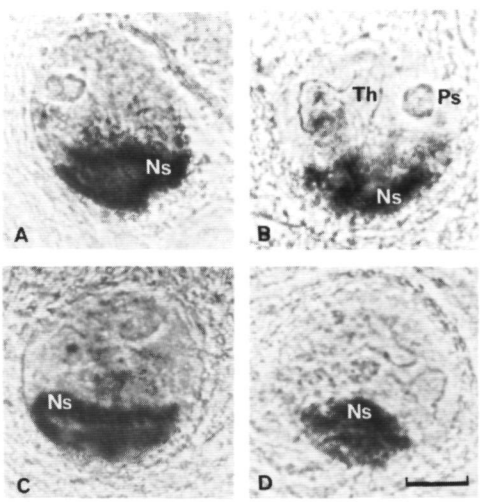

Fig. 3, A – D. Transcription of ay1 and *gypsy* is unaffected in a sterile allele of gene *Q* forming a *Nooses* loop pair of normal morphology. Transcript *in situ* hybridization of digoxigenin-11-UTP-labelled RNA probes for ay1 (**A** and **C**) and *gypsy* (**B** and **D**) to testis tissue of $X/ms(Y)Q4^{ts}$ males. For slides incubated with the same probe all steps in the procedure were identical. The primary spermatocyte nuclei shown in **A** and **B** were from fertile males raised at 18°C, those shown in **C** and **D** were from males grown until eclosion at 18°C, but then shifted to 26°C for 9 days, a treatment which renders them sterile (see Fig. 5). The morphology of the *Nooses* loop pair (*Ns*) in males kept at the restrictive temperature does not differ from that in males grown at the permissive temperature. Also the loop pairs *Threads* (*Th*) and *Pseudonucleolus* (*Ps*) are indicated. Phase contrast. *Bar* represents 10 μm

Northern blot analysis of Nooses *transcripts in males carrying mutant alleles of gene* Q

In addition to hybridization to loop transcripts *in situ*, we also used hybridization to Northern blots of RNA to investigate the amount and size distribution of *Nooses* transcripts in males carrying different mutant alleles of gene *Q* (Fig. 4). Males of the genotypes *T(X;Y)58/0*, *X/Df(YL)50* and *X/Df(YL)51* have the wild-type allele, and therefore serve as positive controls, whereas males of the genotypes *X/ms(Y)Q1* and *X/Df(YS)Q1*, which lack the short arm of the *Y* chromosome, serve as a negative control.

In the lanes containing total RNA from males with the wild-type allele, the *Nooses*-specific PY9 probe hybridizes to a heterogeneous smear of transcripts.

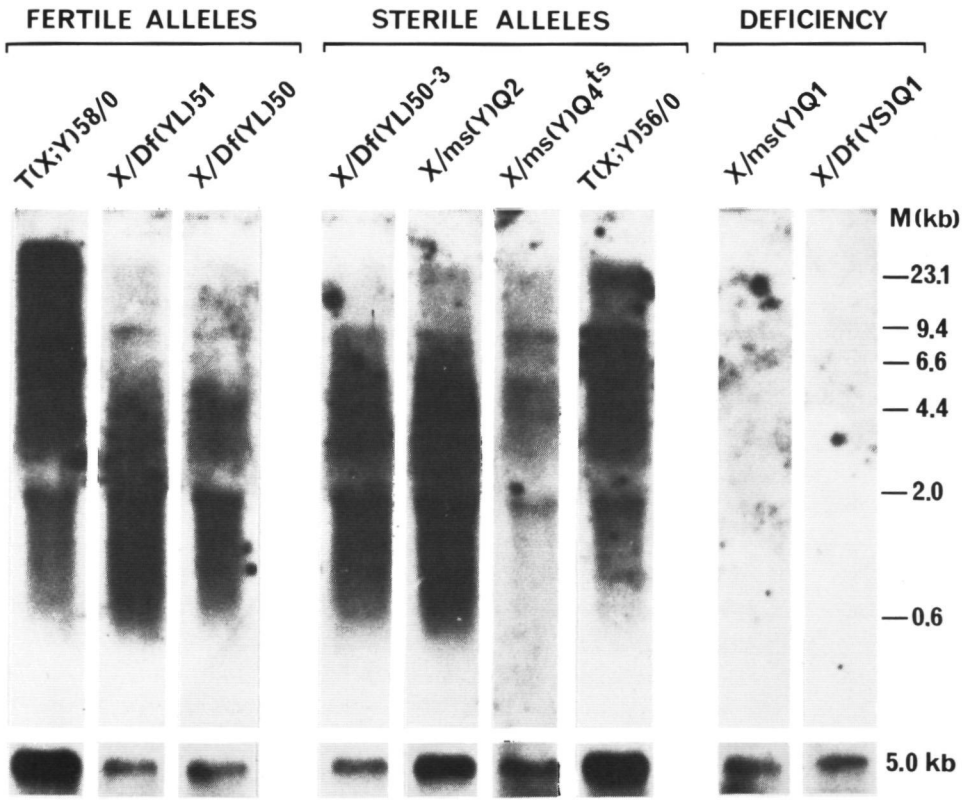

Fig. 4. Northern analysis of total RNA isolated from adult male flies carrying a wild-type allele, a mutant allele, or a deletion of gene *Q*. The blot was hybridized with [^{32}P]-nick translation-labelled PY9 insert, containing only ay1 repeats. Each lane contains about 20 μg RNA. The probe hybridizes to a heterogeneous smear of transcripts. At the level of about 2 kb, the signals become somewhat weaker due to the co-migration of ribosomal RNA. As a control for the amount of testis RNA within the RNA samples loaded, the lower panel shows an exposure of the same blot after hybridization with the cDNA clone cDhT14, which hybridizes to a 5.0 kb RNA species from *D. hydei* testis, but not to transcripts from the somatic parts of adult male flies (Brand and Hennig 1989)

The smear is also seen when isolated testes of wild-type males are used as the source of the RNA (also see for example Vogt et al. 1982; Lifschytz et al. 1983; Trapitz et al. 1988), and also when the DhNo90 *gypsy* element is used as a probe (see Chapter 5). In the lanes containing RNA from *X/ms(Y)Q1* or *X/Df(YS)Q1* males, which both lack gene *Q*, no signals are seen with the PY9 probe, consistent with transcript *in situ* hybridization experiments using ay1 or *gypsy* as *Nooses*-specific probes (Chapters 2, 4 and 5).

Previously, we have interpreted the heterogeneous size distribution of the transcripts containing ay1 and *gypsy* as the combined result of the gradient in transcript size observed in Miller spreads of the *Nooses* loop (Grond et al. 1983), and of degradation of the large (up to 260 kb) transcripts during isolation of the RNA (Vogt et al. 1982; also see Chapter 5). Since this latter factor is likely to vary between the different preparations, the bulk of the hybridizing RNA in flies carrying the wild-type allele of gene *Q* may either be in the range of 5 to 25 kb (as in the first lane of Fig. 4), or in the range of 1 to 10 kb (as in the second and third lanes of Fig. 4). Degradation of the 5 kb size marker is much less compared to the giant loop transcripts. When isolated testes are used for RNA isolation, the bulk of the signal is usually in the upper part of the lane, close to the zone of limiting mobility, indicating that in such preparations, the integrity of large transcripts may be better preserved than those obtained from whole adult males (not shown).

We used four different male-sterile alleles of gene *Q* for Northern analysis. As expected from the transcript *in situ* hybridization experiments described above, *X/Df(YL)50-3* males, forming a morphologically modified *Nooses* loop pair (Fig. 2), and *X/ms(Y)Q4*^{ts} males (raised at 26°C), forming a normally looking *Nooses* loop pair (Fig. 3), display a heterogeneous RNA smear, similar to that seen in the case of the wild type allele. We also found this RNA smear in *T(X:Y)56/0* males, confirming an earlier observation of Lifschytz and Hareven (1985). Males of the genotype *X/ms(Y)Q2*, which also carry a sterile allele forming a *Nooses* loop pair of wild type morphology (not shown), display a similar smear as the wild type. Because in all four cases a weak hybridization of PY9 to RNA in the zone of limiting mobility at the upper part of the blot is seen, large ay1-containing transcripts, that could not be resolved during agarose gel electrophoresis, were present in these RNA preparations. The variation in the amounts of ay1-containing transcripts is within the range seen among the males carrying the wild type allele.

In summary, the analysis of Northern blots indicates that transcription of ay1 repeats is normal in the *Nooses* loop pairs formed by several male-sterile alleles of gene *Q*, irrespective of whether the loop pair is structurally normal or modified. These results are consistent with those obtained by hybridization to loop transcripts *in situ*.

A deletion of gene Q *and a sterile allele that forms a normal* Nooses *loop pair cause similar phenotypes*

The hybridization experiments suggest that transcription of ay1 and *gypsy* is not detectably altered in male-sterile alleles of gene *Q*. Therefore, we investigated

whether the performance of spermatogenesis in males carrying a sterile allele that forms a normal *Nooses* loop pair, is detectably improved compared to males that lack gene *Q* altogether. Fig. 5 shows an overview of spermatid differentiation in *X/ms(Y)Q1* males, which have a deletion of the short arm of the *Y* chromosome and therefore lack fertility gene *Q* (A to D). It also shows spermatid differentiation in sterile *X/ms(Y)Q4^{ts}* males (grown at 26°C for at least 9 days) (E to H). As discussed above, these males have a *Nooses* loop pair of normal morphological appearance.

At the level of the light microscope, the phenotype caused by the conditionally sterile allele at the restrictive temperature is indistinguishable from that caused by the deletion. In both cases meiosis and the early stages of spermatid differentiation are more or less normal, as it was also described for *D. hydei* males lacking the entire *Y* chromosome (Hennig et al. 1974a). The terminal stage of spermatid differentiation in aged males of both genotypes is reached during more

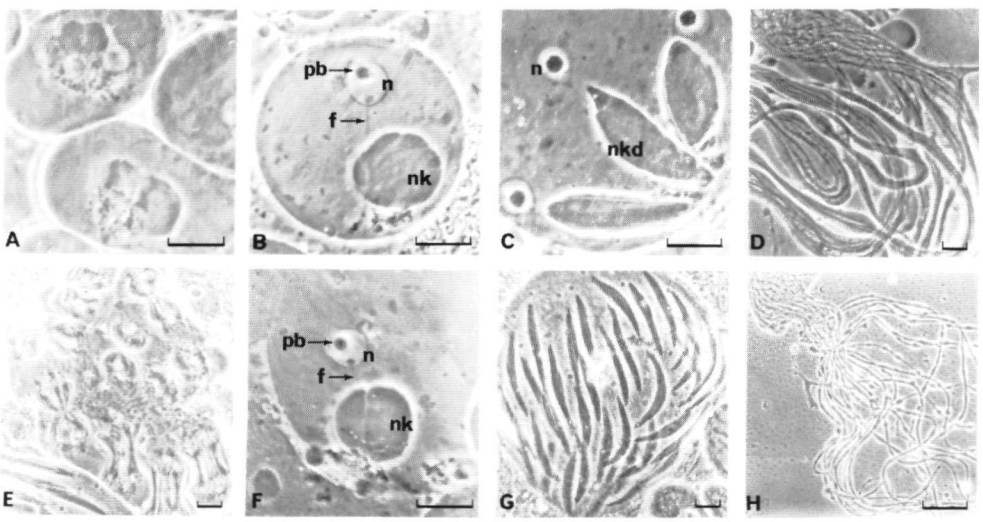

Fig. 5, A – H. Spermatogenesis in males carrying a male-sterile allele of fertility gene *Q* forming a normally shaped *Nooses* loop pair is not different from that in males lacking gene *Q*. **A to D** *X/ms(Y)Q1* males, lacking the short arm of the *Y* chromosome and therefore lacking gene *Q*. **E to H** *X/ms(Y)Q4^{ts}* males, kept at 26°C for 9 days following eclosion. Meiosis (**A, E**) is normal. Also the first stages of postmeiotic spermatid differentiation are not visibly affected, as postmeiotic stage I (PMI) spermatids (**B, F**), contain all structural components of the spermatid. These are a normal round nucleus (*n*) with a single, round protein body (*pb*), a flagellum (*f*) and a normal nebenkern (*nk*). Also subsequent postmeiotic development proceeds normally. **C** PMIII stage, with elongating nebenkern derivative (*nkd*). **G** Cyst of spermatids at the PMIV stage, with normally elongating tails and spindle shaped nuclei. However, the elongated spermatids never reach the individualization stage, and hence, they never attain motility. **D** and **H** Terminal development of spermatids: cysts of elongated spermatids which fail to become individualized (**D**). Subsequently, such cysts start to degenerate (**H**). Descriptions of spermatogenesis in wild-type males of *D. hydei* are given by Grond (1984), Hennig (1985) and Hennig and Kremer (1990). Phase contrast. *Bar* represents 10 μm in **A** to **C** and in **E** to **F**, and 50 μm in **D** and **H**

advanced postmeiotic stages, as elongated spermatids fail to individualize and do not become motile. Subsequently, such cysts start to degenerate.

Thus, compared to a deletion of gene *Q*, a sterile allele forming a *Nooses* loop pair of normal shape does not detectably improve the performance of spermatogenesis at the level of the light microscope. We cannot exclude however, that by a more detailed analysis at the level of the electron microscope, such differences would be detectable.

Discussion

Reliability of the transcript in situ *hybridization procedure in revealing* Nooses *loop morphology*

As shown by genetic studies (Hackstein et al. 1982), there is only one complementation group on the short arm of the *Y* chromosome that can be mutated to male sterility (reviewed in Chapter 1). It identifies, therefore, a single gene (gene *Q*) with a function indispensable for male germ cell differentiation. The lampbrush loop pair *Nooses* is the cytological manifestation of the activity of this gene, since some sterile lesions of gene *Q* also affect the morphology of the *Nooses*, as for example *Df(YL)50-3* (Fig. 3). In addition, alleles that do not form loops at all are always sterile (Leoncini 1977; Hackstein et al. 1982). Thus, the lampbrush loop seems to be an integral part of the fertility gene.

The purpose of the experiments reported here was to investigate whether the transcription of the repetitive DNA sequences in the *Nooses* lampbrush loop are affected in sterile alleles of gene *Q*. The ayl and *gypsy* probes used in these experiments are representative for the majority of the repetitive DNA sequences in the *Nooses* transcription unit, and both sequence types occur throughout the entire loop (Chapters 5 and 7). Therefore, hybridization of either probe to testis squashes directly reveals whether the morphology of *Nooses* loop pair is normal.

The sensitivity of this procedure is illustrated by the following observations. The length of the *Nooses* lampbrush loop pair in wild-type males, as determined by the *in situ* hybridization procedure, is in perfect agreement with earlier light microscopic measurements of Hennig et al. (1974b), and also with the Miller spreading experiments of Grond et al. (1983). All procedures resulted in an estimated loop length of 50 μm. Moreover, even in the light microscope a size gradient of the loop transcripts can be seen, as it was previously observed in the Miller spread (also see Fig. 3 of Chapter 1). Therefore, we are confident that in male-sterile alleles of gene *Q* the morphological details of the *Nooses* loop pair, as far as they can be revealed by light microscopy, are accurately reflected by the labelling patterns of the ayl and *gypsy* probes.

The transcription of repetitive DNA sequences in the Nooses *is required, but not sufficient for the function of fertility gene* Q

The analysis of the sterile allele of gene *Q* on the *Df(YL)50-3* chromosome sug-

gests that its lack of function may be due to an insufficient length of the *Nooses* transcription unit. However, we have shown by *in situ* hybridization (Fig. 3) that the conditionally sterile allele *ms(Y)Q4^{ts}* can form a *Nooses* loop pair of a normal size and cytological appearance, in which both major repetitive sequence components of the loop, ayl and *gypsy*, are transcribed. It is highly unlikely that the temperature shift from 18°C to 26°C causes a decrease in the length of the transcription unit. Although we cannot exclude that at the restrictive temperature, the initiation of loop transcription occurs at a lower rate, we have found that the amount of growing transcripts in the loop, as visualized by *in situ* hybridization, is roughly equivalent to that at the permissive temperature. In addition, the hybridization intensity of an ayl probe to RNA from the sterile *ms(Y)Q4^{ts}* males on Northern blots (Fig. 4) is within the range of variation observed among the different RNA preparations from males carrying the wild-type allele. Thus, the temperature shift from 18°C to 26°C does not seem to have a dramatical effect on the rate of transcriptional initiation in the loop pair formed by the *ms(Y)Q4^{ts}* allele. Quantitative measurements of *Nooses* transcripts, using repetitive DNA sequences that are transcribed in one of the other lampbrush loops as a specific internal standard for transcription during the primary spermatocyte stage, may provide a more accurate assay for the amount of *Nooses* transcripts formed in this sterile allele of gene *Q*.

Also in the case of fertility genes *A* and *C* of *D. hydei*, forming the loop pairs *Threads* and *Pseudonucleolus*, respectively, it has been shown that sterile alleles can form a loop pair of normal cytological appearance (Hackstein et al. 1991). A similar finding has been reported by Bonaccorsi et al. (1988), who studied sterile alleles of each of the three loop-forming fertility genes on the *Y* chromosome of *D. melanogaster*. Thus, it seems that the transcription of the repetitive DNA sequences in the lampbrush loops is not necessarily affected by sterilizing defects in the associated fertility gene.

The comparison of the phenotype caused by a deletion of gene *Q* and that caused by the sterile *ms(Y)Q4^{ts}* allele (Fig. 5), implies that the presence of a normally shaped loop pair as such does not detectably improve spermatid development. A similar conclusion was drawn from the analysis of 42 male-sterile mutations in the loop-forming genes *kl-3*, *kl-5*, or both, on the *Y* chromosome of *D. melanogaster*. Irrespective of whether a given mutation caused the deletion of the loops or left the loops intact, the outer dynein arms in the peripheral microtubule doublets of the axoneme were absent in the sperm of the sterile males carrying these mutations (unpublished observations of R.W. Hardy, cited by Hackstein et al. 1991).

In summary, the phenotype caused by the sterile *ms(Y)Q4^{ts}* allele of fertility gene *Q* is similar to that caused by a deletion of the gene. This sterile allele forms a loop pair which, at the level of the light microscope, is indistinguishable from that formed at the permissive temperature. Since all alleles that do not form the loop pair cause sterility, we conclude that the transcription of the major repetitive loop constituents, ayl and *gypsy*, in the *Nooses* loop pair is required, but not sufficient, for the function of gene *Q* (also see Hennig 1993).

Which loop constituents are affected in male sterile alleles that form a lampbrush loop pair of apparently normal morphology?

The primary spermatocyte nuclei of almost all *Drosophila* species investigated contain lampbrush loop pairs (Hess 1967, Hess and Meyer 1968), and it seems reasonable to assume that, similar to the situation in *D. melanogaster* and *D. hydei*, these loops are formed by fertility genes on the *Y* chromosome. Despite the bewildering variations in loop morphology, even between closely related species (Hess and Meyer 1963a; I. Hennig 1978), the functions of the loop-forming fertility genes seem to be conserved, because at the ultrastructural level, the phenotypes of mutations in two loop-forming genes of *D. melanogaster* are identical to those of two loop-forming genes of *D. hydei* (see Chapter 1 for more details). It is tempting to speculate that the functional similarities between these loop-forming fertility genes are based on conserved DNA sequences.

However, the different families of repetitive DNA sequences found to be transcribed in these fertility genes lack any evolutionary conservation (reviewed in Chapter 1; see Vogt et al. 1986 for ay1 and Chapter 7 for *gypsy*). The lack of evolutionary conservation of the different repetitive DNA sequences transcribed in the loops suggests that the conserved function of the loop-forming fertility genes does not directly depend on the transcription of these particular sequences. Based on the ultrastructural analysis of *D. melanogaster* males lacking gene *kl-5* (Hardy et al. 1981), and on the comparative analysis of testis proteins of wild-type males and males carrying mutant alleles of *kl-5* (Goldstein et al. 1982), it has been postulated that this loop-forming gene encodes a dynein protein of the sperm axoneme. Recently, Gepner and Hays (1993) confirmed these observations by showing that the coding sequences for a dynein β-heavy chain variant map to the region of the *Y* chromosome containing the *kl-5* gene.

Whether the coding sequences of dynein are located within loop pair *A*, formed by *kl-5*, awaits further elucidation, as will be discussed in Chapter 9. From our sequencing studies we have so far no evidence for the presence of protein coding sequences in any of the loop-forming genes of *D. hydei*. However, the availability of temperature-sensitive alleles of certain loop-forming genes of *D. hydei* (Leoncini 1977), including the $ms(Y)Q4^{ts}$ allele of gene *Q*, strongly suggests that these fertility genes also encode a protein. With respect to gene *Q*, this hypothesis would be consistent with our finding that in the light microscope and on Northern blots, the transcription of repetitive sequences without protein coding potential, such as ay1 and *gypsy*, is not detectably affected in the temperature-sensitive allele. The identification of the molecular defect in this allele requires the application of more sensitive techniques than those applied here, and it may even be necessary to reconstruct an entire loop in a set of overlapping clones of genomic DNA.

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CHAPTER 9

General discussion–The mystery continues

The work presented in this thesis has answered several questions concerning the organization of the repetitive DNA sequences within the short arm of the *Y* chromosome of *D. hydei*, and within the *Nooses* lampbrush loop pair. These answers will be summarized and discussed in this chapter. However, the present work provides no direct evidence for the function of fertility gene *Q*, forming the *Nooses*, and also the question why the lampbrush loops are formed remains unanswered.

1 The organization of DNA sequences on the short arm of the *Y* chromosome

The present work was undertaken to construct a contig of the 260 kb of *Y* chromosomal DNA transcribed in the lampbrush loop pair *Nooses* as an ordered set of overlapping genomic clones. The complete reconstruction of the transcribed DNA of the loop as such has not yet been possible. The major reason is that the short arm of the *Y* chromosome harbors much more repeats of the *Nooses*-specific ayl family of repetitive DNA sequences than anticipated from the previous studies of Vogt and Hennig (1983, 1986a,b). Based on the analysis of Southern blots of genomic DNA, these authors concluded that most, if not all, repeats of the ayl family are located within the 260 kb-long *Nooses* transcription unit, and, in addition, that the combined length of all ayl repeats was not more than 80 kb.

As shown in Chapters 2 and 3, these conclusions are not correct, since, using ayl repeats to screen genomic libraries prepared from wild-type flies under non-stringent conditions, more than 1000 kb of genomic DNA could be isolated in lambda and cosmid clones, each containing either ayl repeats, or repeats of the related, but slightly diverged, *Y*-specific YsI family of repetitive DNA sequences. The clones could be assigned to three classes. Approximately 300 kb of genomic DNA contained ayl repeats and additional, unrelated repetitive DNA sequences with copies at other chromosomes (*Y*-associated sequences), some of which hybridized to transcripts of the *Nooses* loop pair *in situ*. At least 300 kb of DNA contained only ayl repeats, and approximately 400 kb of DNA contained only YsI repeats. There were no indications that the isolated DNA fragments were artefacts of the cloning procedure, as discussed in Chapter 2.

The locations of each of these classes of clones on the short arm of the *Y* chromosome could be mapped by *in situ* hybridization to metaphase and interphase chromosomes (Chapter 3). It appeared that the clones containing only ayl repeats are located in a large cluster of presumably uninterrupted ayl repeats, proximal to the position of the *Nooses* loop pair (which was defined by the hybridization of transcribed *Y*-associated DNA sequences, see below). Hybridization of ayl- and YsI-specific probes under stringent conditions to Southern blots of genomic DNA showed that the repeats of both *Y*-specific families are not intermingled (Chapters 2 and 4). Consistent with this conclusion, *in situ* hybridization to metaphase and interphase chromosomes revealed that the YsI repeats are located in an additional megabase cluster, proximal to the ayl megabase cluster (Chapter 3).

These results, which were based on the analysis of chromosomes and genomic DNA from wild-type male flies, confirm those of Trapitz et al. (1992). These authors

used genomic DNA from a *D. hydei* cell line containing the *Y* chromosome, and they concluded that the *Y* chromosome carries an 815-920 kb cluster of uninterrupted ayl repeats and a 630-710 kb cluster of uninterrupted Ysl repeats. Together with the finding that the Ysl sequences do not hybridize to *Nooses* transcripts under stringent conditions (Chapter 4), the localization studies imply that the *Nooses* DNA does not contain repeats of this family.

Using *Y*-associated sequences that hybridized to *Nooses* transcripts (Chapter 2) for *in situ* hybridization to metaphase and interphase chromosomes, the position of the *Nooses* loop pair, and hence of gene *Q*, was mapped distal to the large clusters of uninterrupted Ysl and ayl repeats (Chapter 3). Both ayl and the transcribed *Y*-associated sequences of *D. hydei* are co-transcribed in a *Nooses*-like loop pair of the closely related species *D. eohydei* (Chapter 5). Since this species does not have the megabase clusters of homogeneous ayl and Ysl repeats (and probably even lacks Ysl altogether, see Chapter 4), it can be concluded that, in *D. hydei*, both these clusters are probably not transcribed, and, in addition, that they are of a more recent evolutionary origin than gene *Q*. From this it follows that they are probably of no relevance for the expression of the *Nooses* loop pair, nor for the function of the associated fertility gene *Q*. A limitation to this statement is that it cannot be completely excluded that a small part of the uninterrupted ayl repeats is transcribed in the loop (see next section).

As discussed in Chapter 1, there is no evidence for the presence of fertility genes other than gene *Q* on the short arm of the *D. hydei Y* chromosome. If such an additional gene exists, it has a much lower mutation frequency than gene *Q*. Since the *Nooses* lampbrush loop, formed by gene *Q*, has a size of not more than 260 kb, it seems that a large part of the short arm, with an estimated length of 6000 kb, consists of DNA sequences that are not transcribed in the primary spermatocyte nucleus. The only other exception is the cluster of ribosomal RNA genes at the distal end of the short arm, with an estimated size of 550 kb (Meyer and Hennig 1974; Hennig et al. 1975; U. Schäfer and Kunz 1975). Thus, the large clusters of ayl and Ysl sequences occupy a considerable portion of the genetically inert DNA of the short arm, and the sequence complexity of this DNA is limited.

2 The reconstruction of the *Nooses* transcription unit

The function of the loop-forming fertility gene *Q* is still competely unknown. The reconstruction of the entire loop-forming transcription unit was attempted to gain access to the molecular basis of this function. As a first step towards this end, DNA sequences that are transcribed in the *Nooses* lampbrush loop pair were characterized in considerable detail. As summarized in Tables 1 and 2, one plasmid clone from earlier work by Vogt and Hennig (1983, 1986a,b), and four lambda clones and three cosmid clones, originating from the present work, have been identified as potential segments of the lampbrush loop. These clones do not overlap, and they may represent eight separate sections of the *Nooses* transcription unit. Their analysis allowed the definition of four criteria to test whether a clone of genomic DNA represents a segment of the *Nooses* transcription unit.

Table 1. Clones of genomic DNA representing potential segments of the *Nooses*

clone[1]	length (kb)	ayl DNA (kb)	*Y*-associated DNA (kb)	*gypsy* DNA (bp)
MY3	2 5	1 1	1 4	927
DhNocos7[2]	33 4	30 8	2 6	927
DhNo86	11 0	3 5	7 5	4543
DhNo90[3]	13 5	7 3	6 2	5026
DhNo87	17 2	11 1	6 1	5026
DhNo52	18 6	12 3	6 3	5026
DhNocos18	36 2	14 3	21 9	5026
DhNocos6	22 2	18 1	4 1	283
	Σ 154 6 kb (100%)	98 4 kb (64%)	56 2 kb (36%)	26784 bp
fraction of 260 kb loop	59.4%	37.8%	21.6%	10.3%

Notes

The complete DNA sequence of clone MY3 is described in detail by Vogt and Hennig (1986b) all other clones have been described in the previous chapters

A complete copy of MY3 is present in clone DhNocos7, as shown both by comparison of restriction maps and by partial sequence analysis of the copy in DhNocos7 (Chapter 2) but both copies of MY3 are not identical

As described in Chapter 2, clone classes DhNo90 DhNo87, DhNo52 and DhNocos18 have very similar restriction maps Although the orientation of *gypsy* and ayl has only been determined in the case of DhNo90 (Chapter 5), it is assumed that the other three classes have similar arrangements

(i) The clone should contain repeats of the *Nooses*-specific ayl family

(ii) The clone should contain additional, unrelated DNA sequences, that also hybridize to transcripts of the *Nooses*

(iii) At least some of these additional, unrelated DNA sequences should be represented by fragments of the *gypsy* retrotransposon

(iv) The orientation of the *gypsy* sequences relative to the transcribed strand of ayl in the clone should be such that only the coding strand of *gypsy* can be present in the loop transcipts

These operational criteria are based on three observations First, the DNA sequence content of the eight genomic clones listed in Table 2, reveals an intimate association between ayl and *gypsy* sequences (Chapters 2 and 5) Second, we observed a strong and specific hybridization of "antisense" probes containing either of these two sequences to transcripts of the *Nooses*, both on Northern blots of testis RNA and *in situ* (Chapters 2, 4 and 5) Third, the simultaneous *in situ* hybridization of both ayl and *gypsy* probes suggests that both sequence types occur interspersed throughout the entire loop-forming transcription unit, as expected from the organization of both sequences in the eight genomic clones

There are no data based on testis-derived cDNA clones that confirm these observations. Transcripts containing ayl are not polyadenylated (Chapter 5), and therefore cDNA clones corresponding to *Nooses* DNA must be isolated from cDNA libraries constructed from total testis RNA. Because 95% of the RNA from testis is ribosomal RNA, this is difficult, though not impossible, as shown by Papenbrock (1991). However, the short, ayl-containing cDNA clones recovered by this investigator have an average insert size of only 0.4 kb, and therefore such clones reveal no details of the long-range organization of the DNA sequences that are transcribed in the 260 kb-long *Nooses* loop pair.

The detailed analysis of the eight clones listed in Table 1 indicates that the sequence heterogeneity within the loop is rather limited. Approximately two thirds of the loop DNA is composed of ayl repeats, and of the remaining one third, at least half is occupied by fragments of the *gypsy* retrotransposon (Chapter 7). The other half consists of other, unidentified DNA sequences. Altogether, 154.6 kb of genomic DNA has been recovered in these eight clones, possibly representing almost 60% of the *Nooses* transcription unit.

It is not known whether there are regions of the loop without ayl and *gypsy* that are too large to be cloned in lambda or cosmid vectors, and which therefore would escape detection by the employed cloning strategy. As described in Chapter 2, clone DhNocos18, which fulfills all four criteria, contains a 17 kb region with restriction fragments that do not hybridize to ayl nor to *gypsy* probes. Because this region contains the BamHI site used for cloning, it may even be larger in the genomic DNA. However, in six of the other clones that fulfill all four criteria, the segments of DNA that fail to hybridize to ayl and to *gypsy* probes seem to be much shorter, suggesting that long regions without these sequences, such as those in DhNocos18, may only rarely occur within the *Nooses* loop pair.

An additional uncertainty concerns the question whether large regions of the loop are composed of uninterrupted ayl repeats. The screening of BamHI libraries obviously selects for clones containing interspersed ayl repeats. As far as can be

Table 2. Summary of DNA sequences from putative segments of the *Nooses*

clone	sequenced (bp)	ayl (bp)	*gypsy* (bp)	other (bp)
MY3	2502	1142	927	433
DhNo86	6816	241	4543	2032
DhNo90	6675	691	5026	958
DhNocos6	3381	2299	281	801
	Σ 19374	Σ 4373	Σ 10779	Σ 4224
	(100%)	(23%)	(55%)	(22%)
fraction of 260 kb loop	**7.5%**	**1.7%**	**4.2%**	**1.6%**

judged from the restriction maps, a region with restriction fragments that all hybridize to ay1 but not to *gypsy* probes occurs in clone DhNocos7 (Chapter2). The region has a length of 21 kb and it may be composed mainly, or perhaps even exclusively, of ay1 repeats. Moreover, several of the ay1 cDNA clones isolated by Papenbrock (1991), hybridize to 100-400 kb SalI or XbaI DNA fragments on Southern blots of DNA from a *D. hydei* cell line containing the *Y* chromosome. Because SalI and XbaI rarely cleave in ay1 repeats (Chapters 2 and 4) it seems that these large DNA fragments consist mainly, if not exclusively, of ay1 repeats, containing other DNA sequences perhaps only at their ends. Thus, it cannot be excluded that long regions of homogeneous ay1 repeats, such as they occur in DhNocos7, exist within the loop. The simultaneous hybridization of ay1 and *gypsy* probes to *Nooses* transcripts (Chapter 5) has a limited resolving power and does not permit to exclude this possibility. Since the loop-forming transcription unit is bordered at its proximal end by the megabase cluster of uninterrupted ay1 repeats (Chapter 3), it is therefore possible that transcription of the *Nooses* is initiated or terminated within this cluster.

It should also be emphasized that clones of genomic DNA complying with several, but not all, of the four criteria, cannot be classified as a part of the *Nooses*. For example, clone DhNo19 fulfills criteria (i), (ii), and (iii). However, as shown in Chapter 5, the *gypsy* sequences in this clone have both orientations with respect to ay1, which is incompatible with its location within the transcription unit. The existence of clones such as DhNo19, which fulfill all criteria except (iv), may hamper the reconstruction of the *Nooses* transcription unit. All *Y*-associated *gypsy* elements on the short arm are clustered at the cytological position of the *Nooses* loop pair (Chapter 3). Therefore, clones as DhNo19, with a DNA sequence content almost indistinguishable from clones located within the loop, must be located in close vicinity to the loop, either upstream or downstream of the loop-forming transcription unit. From a total of nine clones containing both ay1 and *gypsy* DNA sequences recovered from the screens (Chapter 2), DhNo19 was the only clone with *gypsy* in both orientations relative to ay1 (Chapter 5). Such clones may therefore be relatively rare. In addition, even if a clone fulfills all four criteria, this is not a definitive proof for its location within the loop. Such a proof becomes only possible by the systematic and complete reconstruction of the entire 260 kb of DNA expressed in the *Nooses* loop pair.

How then, can the initiation and termination points of loop transcription be determined? At the moment, we can only speculate about the definition of the "beginning" and the "end" of the *Nooses* transcription unit. The only data that can guide us in these speculations come from *in situ* hybridization experiments to metaphase and interphase chromosomes (Chapter 3). These experiments indicate that the *Nooses* loop pair is flanked distally by genes for ribosomal RNA, and proximally by the megabase cluster of non-transcribed, homogeneous ay1 repeats. Because the *in situ* hybridization experiments reveal that there is only one ay1 cluster, the proximal end of the loop-forming transcription unit may be correlated with a transition from non-interspersed, non-transcribed ay1 repeats to interspersed, transcribed ay1 repeats. It is, however, also possible that the proximal end is located within the homogeneous cluster of ay1 repeats, as discussed

above, or that the proximal end is correlated with a transition from non-transcribed DNA with *gypsy* in both orientations relative to ayl, to transcribed DNA with *gypsy* in one orientation only. This latter criterium may also apply to the distal end of the *Nooses* loop. It is unknown how much DNA separates the distal end from the ribosomal RNA genes on the short arm, which are transcribed in the nucleolus organizer during the primary spermatocyte stage, as shown by Meyer and Hennig (1974) and by Glätzer (1975).

In summary, these considerations show that it is possible to define operational criteria for determining the proximal and distal ends of the *Nooses* transcription unit. We do not know whether the orientation of transcription within the *Nooses* is from the ayl megabase cluster to the rDNA cluster, or opposite.

3 Implications for the function of the lampbrush loop-forming fertility genes

The rest of this chapter is devoted to an interpretation of the data presented in Chapters 2 to 8. What can be learned from these data with respect to the function, the molecular organization, and the evolution of the lampbrush loop-forming fertility gene *Q*? And what can be learned with respect to the functions of the other loop-forming fertility genes in *D. hydei* and in the other species? Two models are presently favoured in the literature to explain this this function. The loop-forming genes may function by protein binding or by protein coding, or perhaps even both (see for discussion Hennig et al. 1989; Hennig 1990, 1993; Hackstein et al. 1991; Gatti and Pimpinelli 1992).

3.1 Do the loop-forming fertility genes function by protein binding ?

Assuming that the clones listed in Table 1 are located in the *Nooses* transcription unit, does the analysis of their DNA sequence content support a role of the loop-forming genes in protein binding?

From the data presented in Chapters 2, 5, and 7, it can be concluded that ayl and *gypsy* together may occupy 80% of the loop DNA. Both types of loop constituents do not have a protein coding potential, lack a conserved DNA sequence, and randomly accumulate point mutations and deletions (see Chapter 6 for ayl, and Chapter 7 for *gypsy*). In addition, together, these two sequences only occur in the lampbrush loop pairs of *D. hydei* and its closest relatives *D. neohydei* and *D. eohydei*, and several species with *gypsy* elements in the genome do not display *gypsy* transcription in their lampbrush loops (Chapter 7). In Chapter 1, the available data on the DNA sequence content of the other lampbrush loop pairs of *D. hydei* have been reviewed. Although it is not in all cases certain whether the particular DNA sequences studied are transcribed as a segment of the loop, the general conclusions from these studies are that all loop pairs are mainly composed of repetitive DNA sequences without evolutionary conservation, and that individual members of a given family are not identical. Thus, DNA sequence data set for the *Nooses* loop pair is fully consistent with the data set for the other loop pairs of *D. hydei*.

The repetitive DNA sequence content of all the loop-forming genes seems to be quite compatible with the proposed protein-binding function of these genes. Although the ayl repeats, which represent two-thirds of the loop transcripts, lack a conserved DNA sequence (Chapter 6), it cannot be excluded that short sequence motifs are sufficiently conserved to enable the binding of a certain protein at many positions along the loop transcripts. It can also not be excluded that similar short sequence motifs occur in the different families of repetitive DNA sequences in the loop-forming genes of *Drosophila* species that lack ayl repeats. The frequent inclusion of transposable elements as "spacers" into the loop-forming transcription units, as for example *gypsy* in the case of the *Nooses*, may serve to regulate the number of protein binding sites, even if such elements are all defective (Hennig 1987b; Hennig et al. 1989). Transposable element insertions not only increase the length of individual transcripts, but also increase the length of the transcription unit, thereby greatly augmenting the number of loop transcripts attached to the loop DNA. In species that lack *gypsy* elements in their loop-forming fertility genes, members of other retrotransposon families may fulfill this purpose.

However, as discussed in the next section, the DNA sequence data do not rule out an additional possibility for fertility gene function: protein coding. In addition, experimental data, derived from the conditionally sterile allele $ms(Y)Q4^{ts}$ of gene *Q*, do not seem to support the protein binding model. As shown in Chapter 8, transcript *in situ* hybridization of ayl and *gypsy* probes reveals that, at the restrictive temperature, both sequences are transcribed in the *Nooses* loop pair formed by the sterile allele. At the level of the light microscope, there are no differences in morphology between the loop pairs formed at the restrictive and at the permissive temperature. Although *in situ* hybridization does not allow a precise quantification of the initiation rate of loop transcription, Northern blots indicate that at the restrictive temperature, the amount of ayl transcripts is approximately normal in males carrying the $ms(Y)Q4^{ts}$ allele. Therefore, as predicted by the protein binding model, the binding of proteins to *Nooses* transcripts in such males cannot be too drastically affected, if it is affected at all. Nevertheless, gene function is destroyed, as the phenotype is similar to that of a deletion of gene *Q* (Chapter 8).

The conclusion from these and other observations is that it is possible to uncouple loop morphology and fertility gene function. Fertility gene function can be destroyed by a mutation *in cis*, but the morphology of the associated loop pair, and by inference, protein binding, is normal as far as can be judged by direct observation in the light microscope, or by immunological detection of loop-specific antigens (Leoncini 1977; Bonaccorsi et al. 1988; Hackstein et al. 1982, 1991; also see Chapter 1). Therefore, the causal relationship between protein binding and fertility gene function is not obvious. Other observations that are difficult to reconcile with protein binding as the only function of the loop-forming fertility genes have been discussed in Chapter 1. Thus, whereas there is no question that the lampbrush loop pairs bind proteins to their transcripts, the functional significance of this phenomenon is not understood.

3.2 Do the loop-forming fertility genes function by protein coding ?

Is there an obvious relation between protein coding and fertility gene function? Gepner and Hays (1993) have shown that DNA sequences from a gene encoding a dynein β-heavy chain are located on the *Y* chromosome of *D. melanogaster*. These sequences were assigned to a region containing the loop-forming fertility gene *kl-5* but no additional fertility genes. The evidence that gene *kl-5* functions by protein-coding is based mainly on the phenotype of deletions and mutations of *kl-5*, which all lead to an absence of the outer dynein arms in the sperm axoneme (Hardy et al. 1981), and on a temperature-sensitive allele of *kl-5*, that seems to interfere with the incorporation of dynein in the axonemal microtubules, but not with dynein accumulation or stability (Goldstein et al. 1982; also see Chapter 1).

Although it has not been shown that the dynein gene is located within the loop pair formed by *kl-5*, nor that sterile mutations of *kl-5* lead to changes in dynein structure, these observations imply that at least one of the loop-forming fertility genes functions by protein coding. Support for a protein coding function of other loop-forming genes comes from temperature-sensitive alleles of these genes, which have been isolated in *D. melanogaster* (Ayles et al. 1973) and in *D. hydei* (Leoncini 1977). One of the temperature-sensitive mutations in *D. hydei* was assigned to gene *Q* (Hackstein et al. 1982).

The hypothesis that the loop-forming genes are protein coding genes suggests an explanation for the phenomenon of "synthetic sterility" (Hackstein et al. 1982) and even allows some speculations about the functions of the encoded proteins. Synthetic sterility refers to the lack of complementation between sterile alleles of different loop-forming fertility genes. For example, *D. hydei* males of the constitution $X/ms(Y)AC1/ms(Y)Q4^{ts}$ are sterile, although they carry a wild-type allele of each *Y* chromosomal fertility gene.

In a series of genetic studies, M.T. Fuller and co-workers have shown that *D. melanogaster* males, heterogeneous for a mutation in the gene for the testis-specific β_2-tubulin at polytene band 85D on chromosome *3*, are sterile if they are also heterogeneous for mutations in an additional gene (Raff and Fuller 1984; Fuller 1986; Regan and Fuller 1988; Hays et al. 1989; Fuller et al. 1989). Males heterozygous for each individual mutation are fertile, but the doubly heterozygous males are sterile. Such mutations in genes at other genomic positions that fail to complement mutant alleles of the β_2-tubulin gene are called "second-site non-complementors". These mutations affect genes encoding proteins that directly interact with β_2-tubulin, but which are not necessarily expressed only during spermatogenesis. Examples are the *nc33* allele of the α-tubulin gene at polytene band 84B on chromosome *3* (Hays et al. 1989), that is expressed in all tissues throughout development (Matthews et al. 1989), and the *nc2* allele of the *haywire* gene at polytene bands 67E-F on chromosome *3*, that probably encodes a general microtubule-associated protein, as the allele also fails to complement certain α-tubulin mutations (Regan and Fuller 1988, 1990).

Thus, the second-site non-complementors identify partially defective constituents of microtubules, that can be utilized for microtubule assembly in the correct stochiometric amounts. However, during spermatogenesis, they act as

structural poisons that interfere with microtubule function (Fuller et al. 1989). In formal genetic terminology, these examples involving the β_2-tubulin gene are cases of synthetic sterility, similar to the cases described by Hackstein et al. (1982) for certain combinations of mutations in different *Y* chromosomal fertility genes. All these cases may have the same molecular basis: the *Y* chromosomal mutations involved in synthetic sterility may also be partially defective alleles of genes encoding microtubule constituents. Indeed, at least one of the loop-forming *Y* chromosomal fertility genes seems to encode a microtubule-associated protein: the dynein-coding gene *kl-5* of *D. melanogaster* (Gepner and Hays 1993). Based on the analysis of phenotypic defects caused by deletions of gene *kl-3* of *D. melanogaster*, and of genes *A* and *N* of *D. hydei*, these genes may encode dyneins as well (see Chapter 1).

In summary, it is concluded that at least those loop-forming genes for which temperature-sensitive alleles have been isolated are protein coding genes. A function in protein coding would explain why wild-type gene function can be lacking in alleles which contain an apparently normal set of lampbrush loop proteins, as they form a loop pair that is normal at the level of the light microscope. It also explains the one loop-one gene relationship, and, assuming that the encoded gene products are microtubule constituents, such as dyneins, it even explains the phenomenon of synthetic sterility. Most importantly, it explains why mutations of the loop-forming fertility gene *A* of *D. hydei* and gene *kl-5* of *D. melanogaster*, that do not share any of the families of repetitive DNA sequences transcribed in the associated loop pairs, both cause the absence of the outer dynein arms in the sperm axoneme. Since a temperature-sensitive allele of gene *Q* of *D. hydei* has been isolated, it is postulated as a working hypothesis that this gene also is a protein coding gene.

4 Implications for the organization of DNA sequences in the loop-forming genes

If the loop-forming fertility genes are protein coding genes, where are the protein coding DNA sequences? Are they distributed throughout the loop-forming transcription units, or are they clustered at the beginning or at the end of the loop, or even outside of the loop? The evidence for each of these possibilities will be discussed in this section, as the location of the proposed exons of gene *Q* has obvious implications for the reconstruction of the *Nooses* loop pair.

On the molecular level, gene *Q* is the most intensively studied fertility gene, but not more than 7.5% of the associated loop-forming transcription unit has been sequenced, and most of the sequences are either *gypsy* or ayl (Table 2). The remaining 1.6% of other, putative DNA sequences from the loop did not contain long open reading frames, nor significant similarities with sequences in the EMBL databases (EMBL Release 35, June 1993), and there was no statistical evidence for the presence of protein coding sequences. For all other loop-forming fertility genes, the sequence data are even more limited. Therefore, the failure to identify the protein-coding sequences within the loop-forming transcription units may simply be explained by the limited sequence information.

Based on the high mutation frequency of the loop-forming genes, on their large physical sizes, and on their regular complementation in genetic experiments, Hackstein et al. (1991) proposed that the molecular organization of the DNA that is transcribed in the loops is similar to that of the human Duchenne Muscular Dystrophy gene (DMD), with extremely large introns, containing the rapidly evolving, repetitive loop constituents, and much smaller exons. The DMD gene encodes a 14 kb mRNA for a membrane-associated muscle protein called dystrophin (see Anderson and Kunkel 1992 for a recent review). The 75 exons of the gene are distributed in more than 2400 kb of genomic DNA, the average intron size therefore being 35 kb, whereas the average exon size is only 190 bp (Koenig et al. 1987; den Dunnen et al. 1989). Mutations in this *X*-linked gene occur at a very high rate, affecting 1 in every 3500 live male births, and one third of all cases is due to new mutations (Moser 1984; Emery 1988). The high spontaneous mutation frequency of about 1 in every 10000 newly born males is attributed to the large physical size of the gene.

Similarly, the high sensitivity to all kinds of mutagens of the loop-forming genes may also be due to their large size. Data on the mutation frequencies of individual genes are sparse, but the available data for *D. hydei* indicate a correlation between lampbrush loop size and mutation frequency. Male-sterile mutations in gene *Q*, with an estimated target size of 260 kb (Grond et al. 1983), are obtained at a rate of approximately 1% of the treated *Y* chromosomes (J.H.P. Hackstein, personal communication). Genes *A* and *C*, which together may occupy 4000 kb of *Y* chromosomal DNA (Hennig 1985), have a combined mutation frequency of almost 5% (Hackstein et al. 1991). It is unknown what fraction of such mutations is caused by point mutations, deletions, duplications, inversions and translocations. In the case of the DMD gene, 60% of the analyzed mutations are caused by deletions of one or more exons, 7% are duplications of exons, and the remaining 33% are point mutations in splice junctions and coding sequences. Since not all deletions are detectable, the actual fraction of deletions may be even larger (Koenig 1987, 1989; den Dunnen et al. 1987, 1989). Most deletion breakpoints are in introns, not in exons. The distribution of the breakpoints is highly non-random, with a hotspot in the largest intron of the gene, which has a size of 160–180 kb (Wapenaar et al. 1988; den Dunnen et al. 1989; Passos-Bueno et al. 1992).

Thus, a large intron/small exon structure of the lampbrush loops is not necessarily incompatible with a high mutation frequency. Similar to the situation in the DMD gene, many mutations of the loop-forming genes may be due to breakpoints in introns, causing the deletion of all exons located between two breakpoints. If the deletions are small relative to the length of the loop-forming transcription unit, they may not cause detectable modifications of lampbrush loop morphology.

However, several argument against an intron-exon structure of the loops have been raised by Hennig (1993). First, there are no indications for splicing of the nascent lampbrush loop transcripts. Miller spreading experiments of transcribed lampbrush loop chromatin of *D. hydei* (Glätzer and Meyer 1981; Grond et al. 1983; de Loos et al. 1984; and unpublished observations of R. Suijkerbuijk and W. Hennig) showed that the loop transcripts have highly complicated secondary structures that

may be incompatible with the removal of introns by splicing. In addition, in the case of the *Nooses*, a gradient of growing, nascent transcripts can be seen along the loop DNA axis (Grond et al. 1983). The splicing of large introns from the loop transcripts would result in the distortion of such gradients. Second, several of the antisera raised by Z. Wu et al. (1991) against components of the splicing machinery in the lampbrush loops of *Xenopus laevis* and the salamander *Notophthalmus viridescens*, have been used for immunological staining of *D. hydei* testis squashes (Hennig 1993). These antisera recognize epitopes of either ribonucleic acid or proteinaceous components of small nuclear ribonucleoproteins (snRNPs). Although both types of snRNP components are highly conserved in eukaryotes (Birnstiel 1988), and although transcription and processing usually take place simultaneously (Beyer and Osheim 1988; Z. Wu et al. 1991), none of the antisera tested was found to decorate any of the *D. hydei* lampbrush loop pairs, whereas they did stain the autosomes in primary spermatocytes (unpublished observations of W. Hennig).

It would seem therefore, that the splicing of exons in the loop-forming fertility genes either occurs at the very beginning of a lampbrush loop-forming transcription unit, or at its very end (Hennig 1993). However, whereas there are no indications for splicing of nascent loop transcripts, there are also no indications that splicing does not occur. The Miller spread of the entire *Nooses* transcription unit (Grond et al. 1983) shows that a gradient of growing transcripts along the DNA axis of the loop is present only in the first half of the transcription unit (see Fig. 3, Chapter 1). In the second half, the length of the transcripts appears to be more or less constant due to their complex secondary structures, obscuring the visualization of splicing in the nascent transcripts. For the other loop pairs of *D. hydei*, which have only been incompletely visualized in the Miller spreads (Glätzer and Meyer 1981; de Loos et al. 1984), it is even more difficult to exclude the occurrence of splicing. Miller spreads of an unidentified loop pair of *D. melanogaster* (Glätzer 1980) do not allow a discrimination between splicing and non-specific breakage of the long loop transcripts during the spreading procedure. Thus, with respect to the question of splicing of widely separated exons in the nascent loop transcripts, the data from Miller spreads are inconclusive.

In addition, the immunological data are inconclusive as well. If the sizes of introns within the loop-forming transcription units are large, splicing requires only a fraction of the time needed to transcribe a certain intron. Assuming that RNA polymerase II proceeds at a speed of 1.1 to 1.4 kb per minute at 25°C (Thummel et al. 1990; Shermoen and O'Farrell 1991; Irvine et al. 1991), the transcription of a 100 kb intron would require more than one hour. Thus, at any moment, not more than one intron may be spliced in a given loop transcript, and in many loop transcripts there will be no splicing at all. Therefore, splicing components may not be sufficiently concentrated in the loops to permit their detection by immunocytochemistry. Similarly, in oocytes of *Notophthalmus*, the numerous, normal loops are decorated by snRNP antisera, but the conspicuous and much larger landmark loops are not (Z. Wu et al. 1991). These landmark loops share many properties with the lampbrush loops of *Drosophila* spermatocytes (Chapter 1), including the complex ultrastructure of their RNP matrices (Angelier et al. 1990), the decoration by antisera raised against proteins from hnRNP com-

plexes (Roth and Gall 1987; Pinol-Roma et al. 1989), and the apparent predominance of repetitive DNA sequences (Penrad-Mobayed et al. 1991).

In conclusion, the experimental data are incomplete and they do not allow us to make final statements about the arrangement of the exons in the lampbrush loop-forming fertility genes. The large physical sizes and the high mutation frequencies of these genes is highly suggestive of an organization comparable to that of the human DMD gene, with small exons and large introns, the large introns containing the repetitive loop constituents. However, it cannot be excluded that the exons are clustered towards one of the ends of loop, and that the high mutation frequency is due to mutational hotspots within the fertility genes. An interesting case in this context is the 400 kb deletion of YLI repeats in the *tube-proximal* allele of the *Threads*-forming fertility gene *A* of *D. hydei* (Trapitz 1992). This deletion is large relative to the 1000 kb length estimate for the *Threads* transcription unit (de Loos et al. 1984). As a consequence, loop morphology is modified, but fertility gene function as such is not affected (Hess 1965a). With the present state of knowledge of the molecular organization of the proposed protein coding DNA sequences in gene *A*, it cannot be decided whether the deleted 400 kb of repetitive DNA sequences are located within a single, large intron, or whether they represent a part of a non-coding region of the transcription unit that is located either upstream or downstream of the protein coding DNA sequences. An additional problem is how the different models for the arrangements of the exons can be combined with models explaining the rapid substitution of the repetitive DNA sequences in the loops during *Drosophila* evolution. This latter question is discussed in the following section.

5 Evolutionary aspects of fertility gene structure

5.1 DNA sequences from lampbrush loops are typical for heterochromatin

In Chapters 6 and 7 a model for the evolution of the loop-forming genes is discussed that was based on the analysis of genomic clones (Chapter 2) thought to represent DNA from the *Nooses* loop pair. It was postulated that loop evolution includes the following events: (i) the selection of a "founder" sequence, such as ay1, for linear amplification into long tandem arrays, (ii) the invasion of such arrays by retrotransposons, such as *gypsy*, (iii) the subsequent co-amplification of both, and perhaps additional types of DNA sequences into repeat units of a larger size, and (iv) the independent accumulation of mutations by the different repetitive subunits.

A prediction of the model is that evolutionary younger loops, as defined by a smaller degree of DNA sequence divergence between the satellite-like loop constituents, also contain less transposable elements. This prediction is fulfilled, as shown by the analysis of the DNA sequences in the *Threads* and the *Nooses* loop pairs of *D. hydei* (Chapters 6 and 7). The model is also supported by studies on the genomic organization of repetitive DNA sequences from *D. melanogaster* heterochromatin that are not located within the *Y* chromosomal fertility genes, such as the *Suppressor-of-Stellate* repeats on the *Y* chromosome (Livak 1984, 1990;

Balakireva et al. 1992), the *Y*-linked *He-T* DNA elements (Young et al. 1983; Traverse and Pardue 1988, 1989; Valgeirsdottir et al. 1990; Danilevskaya et al. 1991, 1993), and the *Responder* repeats in the heterochromatin of autosome *2* (C.-I. Wu et al. 1988; Cabot et al. 1993). All these repetitive DNA sequences have a recent evolutionary origin. Initially, they occurred as tandem arrays, that subsequently became invaded by transposable elements, followed by the amplification of both sequences into larger repeat units. Finally, recent studies of lambda clones containing segments of the *Y* chromosome of the Mediterranean fruit fly, *Ceratitis capitata* (family Tephritidae) also revealed a close linkage of *Y*-specific and *Y*-associated repetitive DNA sequences (Anleiter and Haymer 1992) that is highly reminiscent of the genomic clones described in Chapter 2. Transposable elements have so far not been identified in these clones (D. Haymer, personal communication).

From this short overview it follows that neither the arrangement of the repetitive DNA sequences in the *Y* chromosomal fertility genes, nor their recent evolutionary origin are exceptional when compared to sequences from other heterochromatic regions of the *Drosophila* genome. What are the evolutionary mechanisms that have generated these arrangements of the different repetitive DNA sequences in the loop-forming genes?

5.2 Origin and maintenance of tandemly repeated DNA sequences

The presence of tandemly repeated DNA sequences in the loop pairs suggests that the linear amplification of a "founder" sequence was a primary event in loop evolution. The "founder" sequences of the *Y*-specific loop-forming fertility genes may originate from other chromosomes, as argued by Hareven et al. (1986) for the Y18C1 repeats that are transcribed in the *Clubs* loop pair of *D. hydei*, since in the more ancient species *D. bifurca*, this sequence is exclusively autosomal. The YL1 repeats transcribed in the *Threads* and *Tubular* ribbons most likely originate from the *X* chromosome (Wlaschek et al. 1988). The origin of ay1 is not known. For reasons that are totally obscure, these and other *Y* chromosomal DNA sequences became selected for amplification into tandem repeat arrays and for transcription in loop-forming fertility genes. In this context it is interesting to note that the DYZ1 and DYZ2 families of *Y*-specific, simple repetitive DNA families, which account for 50–60% of the human *Y* chromosome, are located on autosomes in the gorilla and in the chimpansee, the closest relatives of humans (reviewed by Smith et al. 1987; Vogt 1990). Thus, during primate evolution, autosomal DNA sequences have become "founder" sequences for *Y*-specific tandem repeat arrays. Also the DYZ1 and DYZ2 repeat arrays have been subject to mutation of individual subunits, insertion by other, *Y*-associated sequences, and novel rounds of amplification (Smith et al. 1987), a pattern of sequence evolution very similar to that observed for the loop-forming genes of *Drosophila*.

Theoretical work by Charlesworth et al. (1986) and Walsh (1987) implies that large arrays of tandem repeats, located in regions of the genome that are excluded from meiotic recombination, will tend to decrease by the combined result of intrastrand exchange and unequal interstrand exchange. Examples are the satellite-like

repeats in the loop-forming fertility genes of *Drosophila*, and the DYZ1 and DYZ2 repeats on the human *Y* chromosome. The latter are located outside of the pseudoautosomal region, and therefore they are excluded from recombination (Smith et al. 1987). The frequency of intrastrand and unequal interstrand exchanges during the mitotic and meiotic divisions of the male germ cells of *Drosophila* is unknown. Spontaneous sister chromatid exchanges in a ring-*Y* chromosome occur in 0.3% of mitotic divisions in neuroblasts of *D. melanogaster* (Yamamoto and Miklos 1978), and the frequency in gonial cells may be similar. Therefore, amplification mechanisms, which compensate for decreases in array lengths, must play an important role in the evolutionary stability of tandemly organized repetitive DNA sequences.

The nature of such mechanisms is unknown. It is possible, however, that they not only maintain array length, but that they also cause the sudden amplification of certain "founder" sequences within loop-forming fertility genes, and at other positions on the *Y* chromosome as well. This would explain both the lack of evolutionary conservation of the repetitive loop constituents, and the size differences between *Y* chromosomes of closely related species. For example, the *rally* repeats, transcribed in the loop pair *Threads* of *D. hydei* are only found in this species, as they are absent in the sibling species *D. neohydei* (Huijser and Hennig 1987). The megabase clusters of ayl and Ysl repeats of *D. hydei* seem to be completely absent in *D. eohydei* (Chapters 2, 3 and 4), consistent with the size difference between the *Y* chromosomes of both species (Zacharias et al. 1982). The 5' AAGAC 3' repeats, transcribed in the loop pairs formed by fertility genes *kl-5* and *ks-1* of *D. melanogaster* (Bonaccorsi et al. 1990) are not present in the sibling species *D. simulans* (Lohe and Brutlag 1987), which has less highly repetitive DNA sequences in its genome (5% *versus* 21%), and also has a smaller *Y* chromosome than *D. melanogaster* (Lohe and Roberts 1988).

Thus, it seems that the mechanisms that generate and maintain long arrays of tandemly repeated DNA sequences in the loop-forming fertility genes are the same mechanisms that generate and maintain such arrays at other positions in the heterochromatin.

5.3 Preferential insertion of transposable elements in heterochromatin

Whereas transposable elements are clustered in heterochromatic regions of the *Drosophila* genome, such as the centromere-associated heterochromatin (reviewed by Miklos and Cotsell 1990; also see Chapters 2 and 7), and, in *D. hydei*, the *Y* chromosomal regions giving rise to the *Threads* and *Pseudonucleolus* loop pairs (Huijser et al. 1988; S. Lankenau et al. 1994), and the *Nooses* loop pair (Chapters 3 and 5), their density in the euchromatin is much lower. Aquadro (1993) estimates that, on average, there is one transposable element in every 460 kb of DNA in the euchromatin of *D. melanogaster*, and the occupation frequency per euchromatic site, as measured by *in situ* hybridization on polytene chromosomes of different individuals from the same natural population, is only 1%-9% for most families (Charlesworth and Langley 1991; Aquadro 1993). This suggests that, except in cases

of hybrid dysgenesis, the intragenomic spread of such elements is controlled by forces opposing transposition. It has been postulated that high occupation frequencies per euchromatic site give rise to deleterious chromosome rearrangements, such as deletions, by ectopic exchanges between homologous elements at different sites during meiosis (Charlesworth and Langley 1986, 1991; Montgomery et al. 1987, 1991; Ajioka and Hartl 1989). Since heterochromatic regions of the genome are excluded from meiotic recombination, a high density of transposable elements in such regions, as a result of both *de novo* integration and intrachromosomal amplification, is not necessarily deleterious. This may explain the apparent "target-function" of heterochromatin (Hennig 1986) for such self-replicating, transposable elements. The *Y* chromosome clearly is such a preferred target. At least 3% of the *Y* chromosome of the mouse (*Mus musculus*) is composed of a retrovirus-related DNA sequence, which is present in an estimated 100 copies (Philips et al. 1982) and which has become co-amplified together with *Y*-specific DNA sequences (Eicher et al. 1989). M. Steinemann and S. Steinemann (1991, 1992) and M. Steinemann et al. (1993) have even suggested that during the initial evolution of *Y* chromosomes, recurrent insertions of transposable elements are instrumental in the destruction of the genetic activity of the newly evolved *Y* chromosome.

However, the analyses of *Y* chromosomal fertility genes have shown that transposable elements can even insert into arrays of tandem repeats that are transcribed as part of a gene, without causing gene destruction (Huijser et al. 1988; Chapter 5). The retrotransposons within these genes are sufficiently mutated to prevent them from interfering with normal gene function, as shown in Chapter 7 for the *gypsy* elements in the *Nooses*.

It is well known that the phylogenetic distributions of transposable elements in *Drosophila* do not always reflect phylogenetic classifications based on morphological, cytogenetic, or molecular criteria (Kidwell 1993; Capy et al. 1994). Examples are the *I* retroposon (Abad et al. 1989), the *P* element (Daniels et al. 1990a), the *hobo* element (Daniels et al. 1990b; Simmons 1992), the *jockey* retrotransposon (Mizrokhi and Mazo 1990), and the *mariner* element (Maruyama and Hartl 1991a,b). In addition, there is evidence from laboratory experiments for horizontal transmission of the *P* element (Houck et al. 1991) and the *gypsy* retrotransposon (A. Kim, cited by Plasterk 1993). Therefore, the lack of evolutionary conservation of retrotransposons as lampbrush loop constituents may primarily be an attribute of their idiosyncratic phylogenetic distribution. In addition, as shown in Chapter 7, not all *Drosophila* species with *gypsy* elements display its transcription in a lampbrush loop pair, and the same is true for *micropia* (D.-H. Lankenau 1990, 1993; S. Lankenau et al. 1994). The factors that determine whether a certain family of transposable elements becomes a loop constituent are completely unknown, but it is likely that coincidence plays a certain role here.

5.4 Implications for the evolution of the loop-forming fertility genes

An important question is how a proposed function in protein coding, that may be conserved between *D. melanogaster* and *D. hydei*, can be reconciled with the

rapidly evolving repetitive DNA sequences that occupy the bulk of the transcribed DNA of the loops. This question is difficult to answer for two reasons.

First, we do not know the arrangement of the protein coding sequences and the repetitive DNA sequences (Hennig 1993; also see section 4). It would seem that deletions and additions of long arrays of repetitive DNA sequences, such as they apparently occurred during the evolution of the loop-forming genes, are less likely to affect the protein coding function if the exons are concentrated at either the beginning or at the end of the loop-forming transcription units. However, it also possible that repeats of a certain family occupy whole introns within the lampbrush loops. For example, the recently evolved *rally* repeats of *D. hydei*, which form a 400 kb cluster uninterrupted tandem repeats in the loop pair *Threads* (Huijser and Hennig 1987; Trapitz 1992), may all be located within a giant intron. In the sibling species *D. neohydei*, that does not have *rally* repeats, this intron may be absent or it may have a much smaller size.

Second, we do not know the origin of the promoter of the loop-forming fertility genes. It is possible that a retrotransposon insertion provided the initial promoter sequences for lampbrush loop transcription, as discussed in Chapter 7. However, given the conserved protein coding function of the loop-forming genes, it is also possible that the loop promoter corresponds to the promoter of the particular protein coding gene. In this context it is interesting to note that protein coding sequences can transpose to the *Y* chromosome from positions in the euchromatin, and they can even be transcribed in primary spermatocytes, such as a histone H5-like gene of *D. melanogaster*, which evolved to become a transcribed pseudogene (Russell and Kaiser 1993). Its transposition to the *Y* chromosome must have taken place relatively recently, as such sequences are absent on the *Y* chromosome of *D. simulans*. Did this also happen, but much earlier during evolution, in the case of the dynein gene that has been mapped to the region containing fertility gene *kl-5* on the *Y* chromosome of *D. melanogaster* (Gepner and Hays 1993)? This gene evolved into a gene that must be transcribed to produce male fertility, a property ensuring its permanent presence on the *Drosophila Y* chromosome. But why then, did this gene also acquire the property of forming a lampbrush loop when it is expressed during meiotic prophase?

6 Why are the lampbrush loops formed ?

The phylogenetic distribution of lampbrush loops does not help us much in answering this question. In general, lampbrush chromosomes occur in the oocytes of animal species with large, yolky eggs that, following fertilization, undergo rapid cleavage divisions during early embryogenesis (Davidson 1986; Callan 1986). Their presence in many phyla documents the ancient origin of lampbrush chromosomes, but they have also been lost in many lineages, for example in mammals. Thus, lampbrush loop formation is not a prerequisite for performing oogenesis, and *Drosophila* females do not have lampbrush loops. In the meroistic, polytrophic ovaries of flies, there is almost no transcription in the oocyte nucleus, as the RNA of the developing oocyte is provided by the nurse cells, which contain polytene chromosomes (King and Burnett 1959; Bier 1963).

With respect to spermatogenesis, the *Drosophilidae* appear to be the only dipterans, the only insects, and in fact, the only group of animals, where large lampbrush loops are formed (Callan 1986; O. Hess, personal communication). Thus, lampbrush loop formation cannot be a prerequisite for performing spermatogenesis. Different from the situation during oogenesis, only one of the chromosomes is involved in lampbrush loop formation, and only a very small number of genes form a loop pair. In trying to answer the question why these genes form loops, two opposing points of view can be taken.

First, from the discussion in the previous sections, it may seem that the evolution of the DNA sequences that are transcribed in the loop-forming transcription units merely reflects the evolution of DNA sequences that are located in heterochromatin, *i.e.* a rapid turnover of satellite-like DNA sequences, and a preferential occupation by transposable elements. From this point of view, the loop-forming fertility genes appear to be protein coding genes with large introns consisting of rapidly evolving repetitive DNA sequences that become invaded by retrotransposons. During the primary spermatocyte stage, the long transcripts containing these introns become decorated by proteins, resulting in the conspicuous lampbrush loops seen in the light microscope. These proteins may be general RNA-binding proteins, that function in somatic cell types as well (Chapter 1). However, this does not answer the question why the *Y* chromosomal loop pairs are formed. No other protein coding genes in *Drosophila* containing large introns form lampbrush loops, and all other dipterans except the *Drosophilidae* lack large loop-forming fertility genes. It would be very interesting to construct transgenic *D. melanogaster*, carrying an ectopic copy of the dynein gene from the loop-forming fertility gene *kl-5* that does not include the associated satellite DNA sequences. It should be feasible to test whether such a copy, located in the euchromatin and not forming a lampbrush loop, is able to rescue the sterility of males lacking gene *kl-5*.

The other point of view is based on the proposed correlation between lampbrush loop formation during male meiotic prophase and the absence of recombination during male meiosis (Hennig 1993). Among the higher Diptera (suborder Brachycera, the flies), male meiosis occurs without chiasma formation (reviewed by White (1973) and Gehtmann. (1988); for *D. melanogaster* see Cooper 1950).

Indeed, recombination between linked marker genes does not take place in *D. melanogaster* (Morgan 1912, 1914), nor in *Scaptomyza gramium* (also belonging to the family Drosophilidae) (Stalker 1945), *Megaselia scalaris* (Phoridae) (Springer 1958; Mainx 1964), *Musca domestica*, the common house fly (Muscidae) (Sullivan 1961; Milani 1975), *Lucilia cuprina*, the Australian sheep blow fly (Calliphoridae) (Whitten et al. 1975; Foster et al. 1981), and *Ceratitis capitata*, the Mediterranean fruit fly (Tephritidae) (Robinson et al. 1989). Cases of aberrant, so-called "male recombination" in *D. melanogaster* (Hiraizumi 1971; Henderson et al. 1978), are ascribed to high frequencies of chromosome breaks that are caused by transposase enzymes encoded by certain families of transposable elements, such as the *P* element family in the case of *P-M* hybrid dysgenesis (Kidwell et al. 1977; Bingham et al. 1982; Duttaroy et al. 1990). This syndrome of gonadal abnormalities also includes an elevated spontaneous mutation rate. In *D. ananassae* "male recombination" has been known for a long time (see Ashburner 1989 for references), but it only occurs in males of certain strains, in which it is invariably correlated with abnormally high frequencies of spontaneous mutation (Hinton 1983; Tobari and Moriwaki 1983) and chromosome breakage (Matsuda et al. 1983). Therefore, it is most likely due to

transposable element activity, similar to the situation in dysgenic *D. melanogaster*. Cases of "male recombination" in *Musca domestica* (Lester et al. 1979), and *Lucilia cuprina* (Foster et al. 1980) involve strains carrying *Y*-autosome translocations, and since the exchanges frequencies are much lower than in the female, their cause is probably, again, different from regular meiotic recombination. Thus, although several cases of apparent "male recombination" have been reported, it is safe to conclude that there is no regular meiotic recombination in male flies.

On this basis, Hennig (1993) has speculated that the loops "serve as a functional substitute for the synaptonemal complexes normally formed during meiotic prophase". Synaptonemal complexes, such as they are formed during *Drosophila* oogenesis (Rasmussen 1974; also see Carpenter 1975,1979; and Schmekel et al. 1993) have not been detected in electron microscopic studies of spermatogenesis in *D. melanogaster* (Meyer 1960; Rasmussen 1973), but it has been shown that antisera raised against synaptonemal complex (SC) proteins from rat testis (Heyting et al. 1989; Offenberg et al. 1991) also decorate the loops formed by several male fertility genes of *Drosophila*, both in *D. hydei* and in *D. melanogaster* (unpublished observations of C. Heyting and W. Hennig, also see Table 4 in Chapter 1). The hypothesis that the loops function by the binding of SC proteins would elegantly explain why certain fertility genes are expressed as lampbrush loops.

However, additional work is required to substantiate this hypothesis. The genes encoding the structural proteins of the SC have not yet been identified, neither in *Drosophila*, not in any other insect species, and it is still unknown, therefore, whether these genes are expressed at all during *Drosophila* spermatogenesis. Another unresolved matter concerns the generality of the occurrence of lampbrush loops among the insects and among the flies. Certainly, not all major groups of insects have been properly investigated in this respect. Are the *Drosophilidae* the only insects, the only dipterans, and the only flies without meiotic recombination in the male but with large *Y* chromosomal lampbrush loops (see Hess 1967b,1980; Callan 1986)? And do all *Drosophila* species have loops that are stained by antisera raised against SC proteins? Wheras the loops of *D. hydei* are extremely large, the loops of many other *Drosophila* species are not as easily recognized in the light microscope. And how about other flies that lack recombination in the male? The house fly has a similar karyotype and a similar genome size as many species in the subgenus *Drosophila* (Milani 1975), but cytogenetic investigations of spermatogenesis did not reveal evidence for the presence of well-developed, *Drosophila*-like lampbrush loop pairs (Perje 1948). However, since the diameter of primary spermatocyte nuclei of the house fly is only 5 μm, much smaller than the 30–45 μm sizes of *D. melanogaster* and *D. hydei*, these nuclei may simply be too small to allow the detection of loop-like structures by conventional microscopy. Do the antisera raised against SC proteins also decorate intranuclear structures in primary spermatocytes of *Musca* and the other species of flies mentioned above?

Is it possible that these opposing views on fertility gene function can be merged? A "dual function" of the loop-forming fertility genes in protein coding and protein binding has been discussed by Hennig et al. (1989), Hennig (1990) and Hackstein et al. (1991). It is hoped that the reconstruction of the *Nooses* loop pair, as initiated by the present work, helps to resolve the issue of the functional significance of the lampbrush loops.

7 Directions for future research on fertility gene *Q*

As described in this thesis, the reconstruction of fertility gene *Q* has been started by a reconstruction of the associated lampbrush loop pair *Nooses*. Approximately 60% of the 260 kb long *Nooses* loop pair of may have been recovered in one PstI and seven BamHI clones of genomic DNA (see Table 1), that do not overlap. Continuation of this work towards a complete reconstruction of the entire transcription unit in an ordered set of overlapping genomic clones may proceed along the following lines.

(i) Screening genomic libraries, constructed with different restriction enzymes than BamHI or PstI, for clones that overlap or connect the existing clones, using leftmost or rightmost DNA fragments from these clones as a probe. The clones listed in Table 1 are sufficiently heterogeneous to allow the recognition of overlaps (see Chapter 2). Similar to BamHI, the enzymes chosen for library construction should cleave ayl and Ysl repeats infrequently in order to increase the chances that the isolated clones contain *Y*-associated DNA sequences. Suitable enzymes for this purpose are HindIII and BglII (see Trapitz et al. 1992).

(ii) Screening genomic libraries made with the aid of vector systems that allow the cloning of larger DNA segments than the 40 kb capacity limit of cosmid vectors. Yeast Artificial Chromosome (YAC) vectors allow the cloning of many hundred kb of foreign DNA (Burke et al. 1987), and the bacteriophage P1 system of *E. coli* allows the cloning of DNA segments up to 100 kb (Sternberg 1992). Both systems have been succesfully applied in the *D. melanogaster* genome project (Ajioka et al. 1991; Smoller et al. 1991; Hartl et al. 1992). In the case of *D. hydei*, P1 libraries are not available. The feasability of YAC cloning for the molecular analysis of the fertility genes has been investigated by Kurek (1991). In theory, a single YAC clone or three or four P1 clones may suffice to reconstruct the entire *Nooses* loop. The extant clones provide the probes to screen these libraries, which should be preferentially constructed using enzymes as BamHI, PstI, HindIII or BglII, that rarely cleave in ayl and Ysl repeat sequences. Screening conditions must be very stringent to avoid cross-hybridization to Ysl and ayl repeats that are not located within the *Nooses* loop. Although stable YAC clones containing repetitive DNA from the *D. melanogaster Y* chromosome have been described (Danilevskaya et al. 1991), it is not known to which extent YAC and P1 clones containing large inserts, that consist mainly of repetitive DNA sequences, faithfully represent a genuine genomic situation.

(iii) Screening genomic libraries of *D. eohydei*, again using the extant clones as a probe. As shown in Chapter 5, this species has a *Nooses*-like loop pair in which both ayl and *gypsy* are transcribed with the same strand-specificity as in *D. hydei*. Compared to *D. hydei*, this species offers two advantages when attempting the reconstruction of the *Nooses* loop. First, the *Y* chromosome of *D. eohydei* contains much less ayl than that of *D. hydei*, and it does not contain Ysl repeats (Chapter 4; also see Vogt et al. 1986; Hareven et al. 1986). This considerably alleviates the problem of cross-hybridization to DNA sequences that are not located in the loop. Second, the *Nooses*-like loop formed in primary spermatocytes of this species is more compact than that of *D. hydei*, suggesting that the length of the DNA tran-

scribed in this loop is much smaller (Chapter 5). Assuming that the function of fertility gene *Q* is conserved between both species (see Chapter 1), the reconstruction of the *D. eohydei* loop should give the same information on the nature of the DNA sequences of the gene that are mutable to male sterility as the reconstruction of the *D. hydei* loop. P. Vogt and W. Hennig have isolated ayl-containing lambda clones from *D. eohydei*. The analysis of these genomic clones has recently been initiated by Ya Xian Wang and W. Hennig, and as a first result, most of them were found to contain both ayl and *gypsy* DNA sequences.

While the reconstruction of the *Nooses* transcription unit is proceeding, additional efforts should be aimed at the identification of protein coding sequences in the genomic clones representing the loop-forming transcription unit.

(i) Detailed restriction maps should be constructed for all clones which, by their DNA sequence content, represent potential fragments of the *Nooses* transcription unit. Restriction fragments of these clones that neither contain ayl nor *gypsy* must be used as probes on Southern blots of genomic DNA to test whether, under stringent conditions, these fragments represent single copy, *Y*-specific DNA sequences. If this is the case, the fragments should hybridize to genomic DNA from females carrying *T(X;Y)s* containing the short arm of the *Y* chromosome, but not to genomic DNA from wild-type females, nor to genomic DNA from males carrying deletions of the short arm.

In addition, such restriction fragments should be hybridized to Northern blots of polyadenylated RNA from testis in order to test whether they contain DNA sequences that are present in a polyadenylated mRNA species of a specific size. Most importantly, it should be tested by transcript *in situ* hybridization whether such sequences are indeed transcribed in the *Nooses* loop pair, and also whether their transcripts are transported to the cytoplasm, as expected for protein coding sequences.

(ii) A more direct way to identify protein coding sequences employs the polymerase chain reaction (PCR) for the amplification of sequences that flank transcribed ayl repeats from total testis RNA. Primers should correspond to the best conserved sequence domains in the ayl cDNA clones described by Papenbrock (1991) and the ayl repeats from the eight genomic clones listed in Table 1 (see Chapter 7). Only one ayl primer would be needed, since the ayl primer-extended *minus* strand products can be tailed with poly(dC), using oligo(dG) for *plus* strand synthesis. However, this may not be straightforward, as ayl repeats can be interrupted at any position by unrelated sequences, and protein coding sequences may be too far away to be reached by ayl-primed reverse transcription. Any PCR product obtained must be tested similarly as the restriction fragments that do not contain ayl nor gypsy, see (i).

(iii) Restriction fragments and PCR products containing potential protein coding DNA sequences should be used for DNA sequence analysis. Conceptual translations of the identified open reading frames may reveal similarities to known proteins, and if not, statistical methods for identifying protein coding sequences should be applied (see for example Doolittle 1986,1990; Von Heijne 1987). It is not possible to make predictions about the proposed protein. However, it should not be forgotten that mutations of gene *Q* lead to a failure of individualization of the

elongating spermatids (Chapter 1). The product of gene *Q* may directly be involved in elongation, individualization, or coiling of the sperm.

(iv) When protein coding sequences have been identified, predicting a protein that is in one way or the other compatible with the proposed function of gene *Q* in spermatid differentiation, it should be attempted to isolate complete cDNA clones from cDNA libraries prepared from poly-A^+ RNA from testis, and cDNA clones should be used for sequence determination.

(v) PCR can also assist the isolation of protein coding sequences from males carrying mutant alleles of gene *Q*, and for investigating whether other *Drosophila* species contain similar DNA sequences on the *Y* chromosome. For example, it will be important to investigate whether these sequences are located within one of *Y* chromosomal fertility genes of *D. melanogaster*, the only other species where these genes have been extensively characterized at the genetic level.

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CHAPTER 10

Summary – Samenvatting

SUMMARY - The *Y* chromosome of the fruitfly *Drosophila* carries a small number of genes that are essential for male fertility. Some of these genes are expressed as large lampbrush loop pairs during the primary spermatocyte stage of male germ cell development. The available knowledge about the loop-forming genes is summarized and reviewed in **Chapter 1**. Although previous attempts to identify DNA from the lampbrush loops yielded clones that hybridized to loop transcripts, these clones were much smaller compared to the loops and they only contained repetitive DNA sequences. Therefore, it was not certain whether they really originated from the loop-forming genes.

In the present work an unequivocal identification of the transcribed DNA has been attempted by the reconstruction of an entire loop-forming transcription unit in a set of overlapping genomic clones. The lampbrush loop pair *Nooses*, formed by male fertility gene *Q* on the *Y* chromosome of *Drosophila hydei*, was chosen for this purpose. It is the smallest of all *Y* chromosomal lampbrush loops, and, previously, a family of *Y*-specific repetitive DNA sequences had been identified that is specifically transcribed in this loop pair, the ayl family. Most, if not all, members of this family were expected to be located within the loop.

Chapter 2 deals with the cloning of the loop DNA. As this DNA is repetitive, conventional chromosome walking is impossible. Therefore, the strategy was chosen to collect as much DNA as possible from genomic libraries in lambda and cosmid vectors, using ayl repeats as a probe. Unexpectedly, however, more than 1000 kb of ayl-containing DNA was cloned. This is much more than can be accommodated within the 260 kb length of the *Nooses* transcription unit. A classification was achieved which separates the cloned DNA into (i) clones containing ayl and other DNA sequences that have copies on other chromosomes as well (*Y*-associated DNA sequences) (together 300 kb), (ii) clones containing only ayl (more than 300 kb), and (iii) clones containing repeats of the ayl-related Ysl family of repetitive DNA sequences (more than 400 kb).

The following two chapters deal with the identification of the loop segments within this abundance of cloned DNA. In **Chapter 3** this is accomplished by *in situ* hybridization to metaphase and interphase chromosomes. It is shown that *Y*-associated DNA sequences that hybridize to *Nooses* transcripts are clustered within a small region at the distal end of the short arm of the *Y* chromosome. This region contains the loop-forming DNA and defines the position of fertility gene *Q*, since clones containing only ayl or only Ysl repeats, that lack such transcribed *Y*-associated sequences, are located more proximally on the short arm. In **Chapter 4** it is confirmed that the members of the Ysl family are located outside of the *Nooses* transcription unit, since Ysl repeats do not hybridize to loop transcripts under conditions when ayl clearly does.

In Chapters 5, 6 and 7, a description is given of the DNA sequences in potential loop segments. These sequences were taken from four lambda and three cosmid clones that have all the properties of being a part of the DNA that is transcribed in the *Nooses* loop pair. In **Chapter 5** it is shown that these clones contain *Y*-associated retrotransposons of the *gypsy* family, which occur throughout the *Nooses* loop pair. Only the coding strand of *gypsy*, and only a particular strand of ayl are present in the loop transcripts. Assuming that these clones are representative

for the *Nooses* loop, the ayl repeats occupy approximately two-thirds of the 260 kb-long transcription unit, and *gypsy* occupies at least half of the remainder.

The various ayl repeat sequences are described in **Chapter 6**. All repeats analyzed are different due to point mutations, deletions, and duplications, irrespective of whether they are located in the transcription unit. Since the transcribed ayl repeats are not significantly better conserved than nontranscribed ayl or YsI repeats, it is concluded that their DNA sequence content is not under selective pressure.

The analysis of 20 kb of *gypsy* sequences is presented in **Chapter 7**. All *gypsy* elements within the loop are defective and truncated, and they cannot encode proteins, such as reverse transcriptase or integrase. They seem to be degenerating by the random accumulation of point mutations and small deletions. A model is described explaining how these elements became an abundant loop constituent without affecting the function of fertility gene *Q*.

In **Chapter 8** an analysis is presented of the *Nooses* transcripts formed by several male-sterile alleles of fertility gene *Q*.

These findings are discussed in **Chapter 9**. Based on studies of other loop-forming fertility genes of *D. hydei* and *D. melanogaster*, it is postulated that the these genes perform dual functions: a function in protein coding, and a function in protein binding. There are several indications that ayl and *gypsy* are unlikely targets for mutations that interfere directly with the protein coding function. Both types of loop constituents do not have a conserved DNA sequence. They are not found in the lampbrush loops of any other *Drosophila* species except *D. neohydei* and *D. hydei*, the closest relatives of *D. hydei*. In at least one sterile allele of gene *Q*, transcription of ayl and *gypsy* is apparently normal, and the *Nooses* loop pair formed by this allele has a normal morphological appearance in the light microscope (**Chapter 8**). It is unknown whether the exons of the proposed protein coding gene are located within the associated loop.

However, a protein coding function of the loop-forming genes alone does not explain why they form lampbrush loops. Thus, it is possible that the loop-forming genes also perform an additional function in the binding of certain proteins to nascent loop transcripts. The diverged ayl repeat sequences may nevertheless contain the conserved binding sites for such proteins. With respect to gene *Q*, these hypotheses can be tested by completing the reconstruction of the loop-forming transcription unit, either in *D. hydei*, or in *D. eohydei*, which has a much smaller *Nooses*-like loop pair.

SAMENVATTING - Het *Y*-chromosoom van de fruitvlieg *Drosophila* draagt een klein aantal genen dat essentieel is voor de mannelijke vruchtbaarheid. Sommige van deze genen komen tot expressie als grote lampenborstel-loops tijdens het primaire spermatocytenstadium van de zaadcelvorming. In **Hoofdstuk 1** wordt een overzicht gegeven van de tot nu toe verzamelde kennis over de loop-vormende genen. Eerdere pogingen om DNA van de loops te identificeren leverden clonen op die weliswaar hybridiseerden met loop-transcripten, maar die veel kleiner waren dan de loops en die bovendien uitsluitend uit repetitieve DNA-sequenties bestonden. Het kon derhalve niet met zekerheid gesteld worden dat deze clonen inderdaad uit de loop-vormende genen afkomstig waren.

Het doel van het onderzoek, dat in dit proefschrift wordt beschreven, was het bereiken van een ondubbelzinnige identificatie van loop DNA. Hiertoe werd getracht een complete loop te reconstrueren in een geordende set van overlappende genoom-clonen. De keus viel op het lampenborstel-loop paar *Nooses*, dat gevormd wordt door het vruchtbaarheidsgen *Q* op het *Y*-chromosoom van *Drosophila hydei*. Deze loop is de kleinste van alle *Y*-chromosomale lampenborstel-loops. Bij het begin van het onderzoek was reeds een familie van *Y*-specifieke repetitieve DNA-sequenties bekend, de zogenaamde ayl-familie, die alleen in het *Nooses* loop paar getranscribeerd wordt. De meeste, zo niet alle leden van deze familie zouden in de loop gelegen zijn.

Hoofdstuk 2 gaat over het cloneren van het loop DNA. Omdat dit DNA repetitief is, kan een gebruikelijke "chromosoomwandeling" niet gemaakt worden. Er werd daarom gekozen voor het verzamelen van zoveel mogelijk ayl-bevattend DNA uit genoom-bibliotheken in lambda- en cosmide-vectoren. Er kon echter ruim 1000 kb aan ayl-bevattend DNA worden gecloneerd, wat veel meer is dan de lengte van de *Nooses* transcriptie-eenheid, die "slechts" 260 kb meet. Het gecloneerde DNA kon in drie klassen worden ingedeeld: (i) clonen die bestaan uit ayl en andere DNA-sequenties met copieën op andere chromosomen, zogenaamde *Y*-geassocieerde DNA-sequenties (samen 300 kb), (ii) clonen die uitsluitend bestaan uit ayl (meer dan 300 kb), en (iii) clonen die uitsluitend bestaan uit repeats van de aan ayl verwante Ysl-familie van repetitieve DNA-sequenties (meer dan 400 kb).

De volgende twee hoofdstukken gaan over het identificeren van de loopsegmenten in deze overvloed aan DNA. In **Hoofstuk 3** wordt hiertoe *in situ* hybridisatie op metafase en interfase chromosomen gebruikt. *Y*-geassocieerde DNA-sequenties die hybridiseren met *Nooses*-transcripten zijn bij elkaar gelegen, distaal op de korte arm van het *Y*-chromosoom. Dit gebied moet het loop-vormende DNA en dus ook het vruchtbaarheidsgen *Q* bevatten, omdat clonen met uitsluitend ayl-repeats of Ysl-repeats een meer proximale positie op de korte arm innemen. Met betrekking tot de Ysl-repeats wordt deze conclusie bevestigd in **Hoofdstuk 4**: Ysl-repeats hybridiseren niet met *Nooses* transcripten onder stringente condities die wel de hybridisatie van ayl toestaan.

In de volgende drie hoofdstukken worden de DNA sequenties in de potentiële loopsegmenten beschreven. Deze sequenties zijn afkomstig uit vier lambda- en drie cosmide-clonen die aan alle voorwaarden voldoen om voor ligging in de *Nooses* in aanmerking te komen. In **Hoofdstuk 5** wordt aangetoond dat deze clonen *Y*-geassocieerde retrotransposons van de *gypsy*-familie bevatten, die verspreid

voorkomen binnen de hele loop. Alleen de coderende DNA-streng van *gypsy* en alleen één bepaalde DNA-streng van ayl zijn aanwezig in de loop-transcripten. Wanneer verondersteld wordt dat deze clonen representatief zijn voor de *Nooses*-loop, dan nemen ayl-repeats ongeveer twee-derde van de 260 kb lange transcriptie-eenheid in beslag, en *gypsy* tenminste de helft van het resterende derde deel.

De ayl-repeat sequenties worden beschreven in **Hoofdstuk 6**. Alle onderzochte repeats verschillen van elkaar door mutaties, deleties en duplicaties, ongeacht of ze binnen of bulten de transcriptie-eenheid zijn gelegen. De getranscribeerde ayl-repeats zijn niet aanmerkelijk beter geconserveerd dan de niet-getranscribeerde repeats, waaruit kan worden opgemaakt dat de DNA sequenties van de repeats binnen de loop blijkbaar niet onder selectieve druk staan.

De *Y*-geassocieerde *gypsy*-sequenties worden beschreven in **Hoofdstuk 7**. Alle *gypsy*-elementen zijn defect en ingekort, en kunnen niet meer voor eiwitten, zoals reverse transcriptase en integrase, coderen. Het lijkt erop alsof ze steeds verder degenereren ten gevolge van de aanhoudende accumulatie van puntmutaties en deleties. Tevens wordt een model beschreven dat verklaart hoe deze elementen een belangrijk deel van de loop konden innemen zonder de functie van vruchtbaarheidsgen *Q* aan te tasten.

In **Hoofdstuk 8** wordt de transcriptie van ayl en *gypsy* in steriele allelen van gen *Q* geanalyseerd.

Al deze bevindingen worden bediscussieerd in **Hoofdstuk 9**. Op grond van onderzoek aan andere lampenborstel-loop vormende genen van *D. hydei* en ook van *D. melanogaster* wordt gepostuleerd dat deze genen een dubbele functie vervullen: een functie in eiwit-codering en een functie in eiwit-binding. Er zijn verschillende aanwijzingen dat zowel ayl als *gypsy* waarschijnlijk geen doelwit zijn voor mutaties die leiden tot een rechtstreekse verstoring van de eiwit-coderende functie. Immers, beide hebben geen geconserveerde DNA-sequentie, en ze komen niet voor in de lampenborstel-loops van enige andere *Drosophila* soort, behalve *D. neohydei* en *D. eohydei*, de nauwste verwanten van *D. hydei*. Bovendien worden beide sequenties in tenminste één steriel allel van gen *Q* op klaarblijkelijk normale wijze getranscribeerd, en *Nooses* loop-paar dat door dit allel wordt gevormd heeft een normale verschijningsvorm onder het lichtmicroscoop (**Hoofstuk 8**). Het is niet bekend of de exonen van het veronderstelde eiwit-coderende gen binnen de loop gelegen zijn.

Een eiwit-coderende functie op zich kan echter niet verklaren waarom de fertiliteitsgenen een lampenborstel-loop vormen. Het is derhalve mogelijk dat deze genen nog een andere functie vervullen: het binden van eiwitten aan de loop-transcripten. De gedivergeerde ayl-sequenties zouden bindingsplaatsen voor zulke eiwitten kunnen bevatten. Met betrekking tot gen *Q* kunnen deze hypothesen getest worden door het voltooien van de reconstructie van de *Nooses* transcriptie-eenheid, hetzij in *D. hydei* of in *D. eohydei*, waar een kleiner *Nooses*-achtig loop-paar voorkomt.

APPENDIX

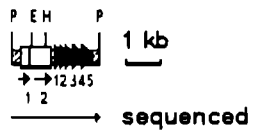
Access key to DNA sequences

This Appendix contains short descriptions of all DNA sequences that were determined during the course of this work For completion, also the MY3 sequence, which has been described previously, has been included, since it was found to contain both *gypsy* and ayl

The following legend refers to all the listed clones

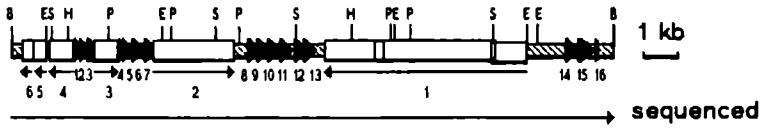
- In the restriction maps, DNA sequences with similarity to the *gypsy* element are indicated by *open rectangles*, sequences with similarity to ayl are indicated by *dark shading*, and unidentified sequences (see below) are indicated by *diagonal hatching*. Individual ayl repeats (as defined by sequence alignment with the original ayl repeat described by Vogt and Hennig (1986a), also see Chapters 4 and 6), are indicated by *black arrowheads*. The orientation of the coding strand of *gypsy* is indicated by an *arrow* underneath each *gypsy* element. The orientation of *gypsy* fragment 1 of clone DhNocos6 has not been determined. Restriction enzyme site abbreviations are *A*, AvaI, *Ac*, AccI, *B*, BamHI, *E*, EcoRI, *H*, HindIII, *P*, PstI, *S*, SalI, *Ss*, SstI.
- The numbers of the ayl and Ysl repeats refer to those used in Chapter 6. For more details, consult this chapter
- The numbers of the *gypsy* elements refer to those used in Chapter 7 For more details, consult this chapter.
- Unidentified DNA sequences are defined as sequences without significant matches when screened against all nucleotide sequences in EMBL Release 35 (June 1993), using the FASTA program of Pearson and Lipman (1988) with ktup = 4

MY3 (2.5 kb) (also see Vogt and Hennig 1986b)



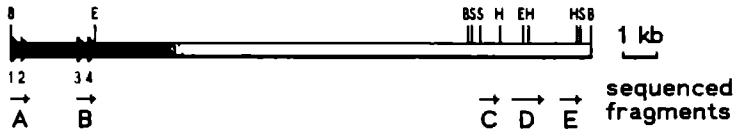
Clone		**MY3**
EMBL accession number.		**X04877**
Identifier key		**DHYLAMP**
Length		2502 basepairs
Features	1-246	unidentified sequence
	247-503	*gypsy* fragment MY3-1
	504-1173	*gypsy* fragment MY3-2
	1174-1187	unidentified sequence
	1188-1340	ayl repeat R1
	1341-1483	ayl repeat R2
	1484-1639	ayl repeat R3
	1640-1973	ayl repeat R4
	1974-2329	ayl repeat R5
	2330-2502	unidentified sequence

DhNo19 (17.0 kb)



Clone		**DhNo19**
EMBL accession number		**X74538**
Identifier key		**DHDHNOQ**
Length		17009 basepairs
Features	1-223	unidentified sequence
	224-620	*gypsy* fragment 19-6
	646-982	*gypsy* fragment 19-5
	983-1062	unidentified sequence
	1063-1758	*gypsy* fragment 19-4
	1763-1919	ay1 repeat R1
	1920-2184	ay1 repeat R2
	2185-2347	ay1 repeat R3
	2348-3012	*gypsy* fragment 19-3
	3013-3049	ay1 repeat R4
	3050-3406	ay1 repeat R5
	3407-3735	ay1 repeat R6
	3736-4020	ay1 repeat R7
	4021-6234	*gypsy* fragment 19-2
	6235-6674	unidentified sequence
	6675-6865	ay1 repeat R8
	6866-7198	ay1 repeat R9
	7199-7541	ay1 repeat R10
	7542-7935	ay1 repeat R11
	7936-8264	ay1 repeat R12
	8265-8537	ay1 repeat R13
	8538-8820	unidentified sequence
	8821-14490	*gypsy* fragment 19-1
	10422-10457	poly-A tail
	14491-15232	unidentified sequence
	15233-15246	putative $tRNA_1^{lys}$ primer binding site
	15246-15258	5' *gypsy* LTR fragment
	15259-15541	unidentified sequence
	15542-15911	ay1 repeat R14
	15912-16382	ay1 repeat R15
	16393-16470	ay1 repeat R16
	16471-17009	unidentified sequence
	16520-16788	86% similarity to nucleotides 229-500 of DhNocos6, fragment A

DhNo55 (16.4 kb)



Clone		**DhNo55, fragment A**
EMBL accession number		**X74887**
Identifier key		**DHDNARCA**
Length		537 basepairs
Features	1-332	ay1 repeat R1
	333-537	ay1 repeat R2

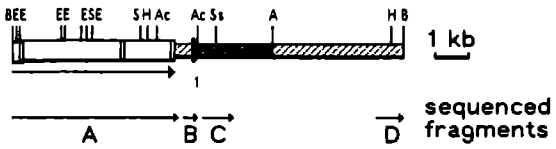
Clone		**DhNo55, fragment B**
EMBL accession number		**X74888**
Identifier key		**DHDNAAY1B**
Length		566 basepairs
Features	1-296	ay1 repeat R3
	297-566	ay1 repeat R4

Clone		**DhNo55, fragment C**
EMBL accession number		**X74889**
Identifier key		**DHDNAUSC**
Length		537 basepairs
Features	1-537	unidentified sequence

Clone		**DhNo55, fragment D**
EMBL accession number		**X74890**
Identifier key		**DHDNAUSD**
Length		877 basepairs
Features	1-877	unidentified sequence

Clone		**DhNo55, fragment E**
EMBL accession number		**X74891**
Identifier key		**DHDNAUSEF**
Length		585 basepairs
Features	1-585	unidentified sequence Note, however, that positions 57 to 202 have 56% similarity with the protease domain of the *17.6* retrotransposon of *D melanogaster* (positions 2532 to 2676 of *17.6* as determined by Saigo et al [1984])

DhNo86 (11.0 kb)



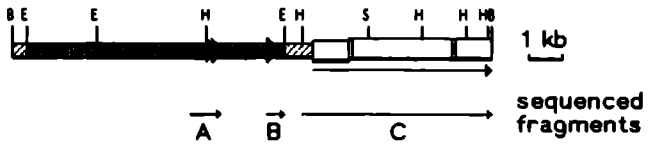
Clone		**DhNo86, fragment A**
EMBL accession number		**X74539**
Identifier key		**DHDHNO86Q**
Length		4652 basepairs
Features	1-4543	*gypsy* fragment 86
	4544-4652	unidentified sequence

Clone		**DhNo86, fragment B**
EMBL accession number		**X74540**
Identifier key		**DHAY1R**
Length		454 basepairs
Features	1-192	unidentified sequence
	193-433	ay1 repeat R1
	434-454	unidentified sequence

Clone		**DhNo86, fragment C**
EMBL accession number		**X74541**
Identifier key		**DHQUS**
Length		921 basepairs
Features	1-921	unidentified sequence

Clone		**DhNo86, fragment D**
EMBL accession number		**X74542**
Identifier key		**DHUSQ**
Length		789 basepairs
Features	1-789	unidentified sequence
	473-608	76% similar to reverse complement of nucleotides 409-546 of DhNo90, fragment B

DhNo90 (13.5 kb)

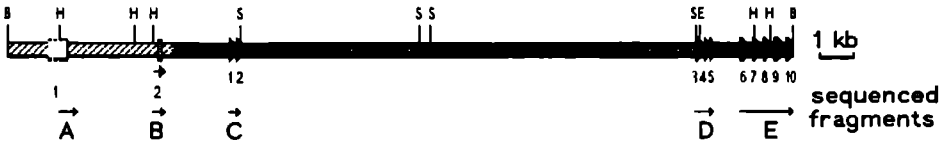


Clone		**DhNo90, fragment A**
EMBL accession number		**X74536**
Identifier key		**DHQAY1**
Length		883 basepairs
Features	1-544	unidentified sequence
	545-656	ay1 repeat R1
	657-749	ay1-like sequence with many alignment gaps
	750-869	ay1 repeat R2
	870-883	unidentified sequence

Clone		**DhNo90, fragment B**
EMBL accession number		**X74537**
Identifier key		**DHQAYRC**
Length		546 basepairs
Features	1-22	unidentified sequence
	23-72	ay1-like sequence
	73-388	ay1 repeat R3
	389-546	unidentified sequence
	409-546	76% similarity to reverse complement of nucleotides 473-608 of DhNo86, fragment D

Clone		**DhNo90, fragment C**
EMBL accession number		**X74543**
Identifier key		**DHDHNO90Q**
Length		5246 basepairs
Features	1-220	unidentified sequence
	221-5246	*gypsy* fragment 90

DhNocos6 (22.2 kb)



Clone		**DhNocos6, fragment A**
EMBL accession number		**X74882**
Identifier key		**DHDNAQG**
Length		500 basepairs
Features	1-228	*gypsy* fragment cos6-1
	229-500	unidentified sequence, 86% similarity to nucleotides 16520-16788 of DhNo19

Clone		**DhNocos6, fragment B**
EMBL accession number		**X74883**
Identifier key		**DHDNAQGL**
Length		403 basepairs
Features	1-135	unidentified sequence
	136-188	*gypsy* fragment cos6-2
	189-403	unidentified sequence

Clone		**DhNocos6, fragment C**
EMBL accession number		**X74884**
Identifier key		**DHDNAAY1**
Length		369 basepairs
Features	1-18	unidentified sequence
	19-289	ay1 repeat R1
	290-369	ay1 repeat R2

Clone		**DhNocos6, fragment D**
EMBL accession number		**X74885**
Identifier key		**DHDNAAY1D**
Length		549 basepairs
Features	1-265	ay1 repeat R3
	266-503	ay1 repeat R4
	504-549	ay1 repeat R5

Clone		**DhNocos6, fragment E**
EMBL accession number		**X74886**
Identifier key		**DHDNAAY1E**
Length		1560 basepairs
Features	1-282	ay1 repeat R6
	283-665	ay1 repeat R7
	666-968	ay1 repeat R8
	969-1345	ay1 repeat R9
	1346-1399	ay1 repeat R10
	1400-1560	unidentified sequence

Ysl repeats from clone **DhNo255**

Accession number	Identifier key	Length (bp)	Code (Chapter 6)
X75055	**DHREP1**	530	**DhNo255S7**
X75056	**DHREP2**	562	**DhNo255PROBE**
X75057	**DHREP3**	514	**DhNo255S6**
X75058	**DHREP4**	508	**DhNo255S2**
X75059	**DHREP5**	504	**DhNo255S1**
X75060	**DHREP6**	413	**DhNo255S4**
X75061	**DHREP7**	329	**DhNo255S12**
X75062	**DHREP8**	165	**DhNo255S10**
X75063	**DHREP9**	119	**DhNo255S13**
X75064	**DHREP10**	97	**DhNo255S23**
X75065	**DHREP11**	68	**DhNo255S16**

Ysl repeats from clone **DhNo327**

Accession number	Identifier key	Length (bp)	Code (Chapter 6)
X75066	**DHREP12**	394	**DhNo327S4**
X75067	**DHREP13**	248	**DhNo327S2**

REFERENCES

Abad PC, Vaury C, Pélisson A, Chaboissier M, Busseau I, Bucheton A (1989) A long interspersed repetitive element - the I factor of *Drosophila teissieri* is able to transpose in different *Drosophila* species Proc Natl Acad Sci USA 86 8887-8891

Ajioka JW, Hartl DL (1989) Population dynamics of transposable elements. In Berg DE, Howe MM (eds) Mobile DNA American Society for Microbiology, Washington DC, pp 939-958

Ajioka JW, Smoller DA, Jones RW, Carulli JP, Vellek AEC, Garza D, Link AJ, Duncan IW, Hartl DL (1991) *Drosophila* genome project one-hit coverage in yeast artificial chromosomes Chromosoma 100, 495-509

Alberola TM, de Frutos R (1993) *Gypsy* homologous sequences in *Drosophila subobscura* (*gypsyDS*) J Mol Evol 36 127-135

Amersham (1984) M13 cloning and sequencing handbook

Amos LA, Amos WA (1991) Molecules of the cytoskeleton. Macmillan, London

Anderson MDS, Kunkel LM (1992) The molecular and biochemical basis of Duchenne muscular dystrophy. Trends Biochem Sci 17 289-292

Angelier N, Bonnanfant-Jais M-L, Herberts C, Lautredou N, Moreau N, N'Da E, Penrad-Mobayed M, Rodriguez-Martin M-L, Sourrouille P (1990) Chromosomes of amphibian oocytes as a model for gene expression significance of lampbrush loops Int J Dev Biol 34 69-80

Anleitei JE, Haymer DS (1992) Y enriched and Y specific DNA sequences from the genome of the Mediterranean fruitfly, *Ceratitis capitata*. Chromosoma 101 271-278

Aquadro CF (1993) Molecular population genetics of *Drosophila* In Oakeshott J, Whitten MJ (eds) Molecular approaches to fundamental and applied entomology, Springer, Berlin Heidelberg New York Tokyo, pp 222-266

Arkhipova IR, Mazo AM, Cherkassova VA, Gorelova TV, Schuppe NG, Ilyin YV (1986) The steps of reverse transcription of *Drosophila* mobile dispersed genetic elements and U3-R-U5 structure of their LTRs Cell 44 555-563

Arkhipova IR, Ilyin YV (1991) Properties of promoter regions of mdg1 *Drosophila* retrotransposon indicate that it belongs to a specific class of promoters EMBO J 10 1169-1177

Ashburner M (1989) *Drosophila* A laboratory handbook Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Awgulewitsch A, Bünemann H (1986) Isolation of Y-chromosomal repetitive DNA sequences of *Drosophila hydei* via enrichment of chromosome-specific sequences by heterogeneous hybridization between female and male DNA J Biochem Biophys Meth 12 37-50

Ayles GB, Sanders TG, Kiefer BI, Suzuki DT (1973) Temperature-sensitive mutations in *Drosophila melanogaster*. XI Male sterile mutants of the Y chromosome Dev Biol 32, 239-257

Baimai V, Sene FM, Pereira MAQR (1983) Heterochromatin and karyotypic differentiation of some neotropical cactus-breeding species of the *Drosophila repleta* species group Genetica 60 81-92

Bairati A (1967) Struttura ed ultrastruttura dell' apparato genitale maschile di *Drosophila melanogaster* Meigen I Il testiculo Z Zellforsch Mikrosk Anat 76 56-99

Balakireva MD, Shevelyov YY, Nurminsky DI, Livak KJ, Gvozdev VA (1992) Structural organization and diversification of Y-linked sequences comprising *Su(Ste)* genes in *Drosophila melanogaster* Nucleic Acids Res 20 3731-3736

Berendes HD (1963) The salivary gland chromosomes of *Drosophila hydei* Sturtevant Chromosoma 14 195-206

Berg DE, Howe MM (1989) Mobile DNA. American Society for Microbiology, Washington, DC

Beridze T (1986) Satellite DNA Springer-Verlag, Berlin Heidelberg New York

Beverly SM, Wilson AC (1984) Molecular evolution in *Drosophila* and the higher diptera II A time scale for fly evolution J Mol Evol 21 1-13

Beyer AL, Osheim YN 1988 Splice site selection, rate of splicing, and alternative splicing on nascent transcripts Gen Devel 2 754-765

Bialojan S, Falkenburg D, Renkawitz-Pohl R (1984) Characterization and developmental expression of β tubulin genes in *Drosophila melanogaster* EMBO J 3: 2543-2548

Bier K (1963) Synthese, interzellulärer Transport und Abbau von Ribonucleinsaure im Ovar der Stubenfliege *Musca domestica* J Cell Biol 16: 436-440

Biessmann H, Mason JM, Ferry K, d'Hulst M, Valgeirsdottir K, Traverse KL, Pardue M-L (1990) Addition of telomere-associated HeT DNA sequences "heals" broken chromosome ends in *Drosophila*. Cell 61: 663-673

Biessmann H, Champion LE, O'Hair M, Ikenaga K, Kasravi B, Mason JM (1992) Frequent transpositions of *Drosophila melanogaster* HeT–A transposable elements to receding chromosome ends. EMBO J 11: 4459-4469

Bingham PM, Kidwell MG, Rubin GM (1982) The molecular basis of *P-M* hybrid dysgenesis: the role of the *P* element, a *P*-strain-specific transposon family. Cell 29: 995-1004

Bingham PM, Zachar Z (1989) Retrotransposons and the *FB* transposon from *Drosophila melanogaster*. In Berg DE, Howe MM (eds) Mobile DNA. American Society for Microbiology, Washington DC, pp 485-502

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for the screening recombinant plasmid DNA. Nucleic Acids Res 7: 1513-1523

BirnstielML (1988) Structure and function of major and minor small ribonucleoprotein particles. Springer, Berlin

Boeke JD, Corces VG (1989) Transcription and reverse transcription of retrotransposons. Annu Rev Microbiol 43: 403-434

Bonaccorsi S, Pimpinelli S, Gatti M (1981) Cytological dissection of sex chromosome heterochromatin of *Drosophila hydei*. Chromosoma 84: 391-403

Bonaccorsi S, Pisano C, Puoti F, Gatti M (1988) *Y* chromosome loops in *Drosophila melanogaster*. Genetics 120: 1015-1034

Bonaccorsi S, Gatti M, Pisano C, Lohe A (1990) Transcripton of a satellite DNA on two Y chromosome loops of *Drosophila melanogaster*. Chromosoma 99: 260-266

Bonaccorsi S and lohe A (1991) Fine mapping of satellite DNA sequences along the *Y* chromosome of *Drosophila melanogaster*: relationships between satellite DNA sequences and fertility factors. Genetics 129: 177-189

Bownes M (1990) Preferential insertion of P elements into genes expressed in the germ-line of *Drosophila melanogaster*. Mol Gen Genet 222: 457-460

Brand RC, W Hennig (1989) An abundant testis RNA species shows sequence similarity to *Y* chromosomal and other genomic sites in *Drosophila hydei*. Mol Gen Genet 215: 469-477

Bridges CB (1916) Non-disjunction as proof of the chromosome theory of heredity. Genetics 1: 1-52; 107-163

Brosseau GE Jr (1960) Genetic analysis of the male fertility factors on the *Y* chromosome of *Drosophila melanogaster*. Genetics 45: 257-274

Brutlag D, Fry K, Nelson T, Hung P (1977) Synthesis of hybrid bacterial plasmids containing highly repeated DNA. Cell 10: 509-519

Burke DT, Carle GF, Olson MV (1987) Cloning of large segments of exogenous DNA into yeast artificial chromosome vectors. Science 236: 806-812

Cabot EL, Doshi P, Wu M-L, Wu C-I (1993) Population genetics of tandem repeats in centromeric heterochromatin: unequal crossing over and chromosomal divergence at the *Responder* locus of *Drosophila melanogaster*. Genetics 135: 477-488

Caccone A, Gleason JM, Powell JR (1992) Complementary DNA-DNA hybridization in *Drosophila*. J Mol Evol 34: 130-140

Callan HG (1982) Lampbrush chromosomes. Proc R Soc London Ser B 214: 417-448

Callan HG (1986) Lampbrush chromosomes. Springer, Berlin Heidelberg New York Tokyo

Callan HG (1987) Lampbrush chromosomes as seen in historical perspective. In Hennig W (ed) Structure and function of eukaryotic chromosomes. Results and problems in cell differentiation, vol 14. Springer, Berlin Heidelberg NewYork, pp 5-26

Callan HG and Mcgregor HC (1958) Action of desoxyribonuclease on lampbrush chromosomes. Nature 181: 1479-1480

Campos-Ortega JA, Hartenstein V (1985) The embryonic development of *Drosophila melanogaster*. Springer, Berlin Heidelberg New York Tokyo

Capy P, Anxolabéhère D, Langin T (1994) The strange phylogenies of transposable elements: are horizontal transfers the only explanation? Trends Genet 10: 7-12

Carlson M, Brutlag D (1977) Cloning and characterization of a complex satellite DNA from *Drosophila melanogaster*. Cell 11: 371-381

Carpenter ATC (1975) Electron microscopy of meiosis in *Drosophila melanogaster* females. I. Structure, arrangement, and temporal changes of the synaptonemal complex in wild-type. Chromosoma 51: 157-182

Carpenter ATC (1979) Synaptonemal complex and recombination nodules in wild-type *Drosophila melanogaster* females. Genetics 92: 511-541

Castrillon DH, Gönczy P, Alexander S, Rawson R, Eberhart CG, Viswanathan S, DiNardo S, Wasserman SA (1993) Toward a molecular genetic analysis of spermatogenesis in *Drosophila melanogaster*: characterization of male-sterile mutants generated by single P element mutagenesis. Genetics 135: 489-505

Charlesworth B (1978) Model for evolution of Y chromosomes and dosage compensation. Proc Natl Acad Sci USA 75: 5618-5622

Charlesworth B (1991) The evolution of sex chromosomes. Science 251: 1030-1033

Charlesworth B, Langley CH (1986) The evolution of self-regulated transposition of transposable elements. Genetics 112: 359-383

Charlesworth B, Langley CH, Stephan W (1986) The evolution of restricted recombination and the accumulation of repeated DNA sequences. Genetics 112: 947-962

Charlesworth B, Langley CH (1991) Population genetics of transposable elements in *Drosophila*. In Selander RK, Clark AG, Whittam TS (eds) Evolution at the molecular level. Sinauer Associates, Sunderland, pp 150-176

Chirgwin JM, Przybyla AE, Macdonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294-5299

Clark AG (1990) Two tests of Y chromosomal variation in male fertility of *Drosophila melanogaster*. Genetics 125: 527-534

Cooper KW (1950) Normal spermatogenesis in *Drosophila*. In Demerec M (ed) The biology of *Drosophila*. Hafner, New Haven, Connecticut, pp 1-61

Corces VG, Geyer PK (1991) Interactions of retrotransposons with the host genome: the case of the *gypsy* element of *Drosophila*. Trends Genet 7: 86-90

Daniels SB, Peterson KR, Strausbaugh LD, Kidwell MG, Chovnick A (1990a) Evidence for horizontal transmission of the *P* element between *Drosophila* species. Genetics 124: 339-355

Daniels SB, Chovnick A, Boussy IA (1990b) Distribution of *hobo* transposable elements in the genus *Drosophila*. Mol Biol Evol 7: 589-606

Danilevskaya ON, Kurenova EV, Pavlova MN, Bebehov DV, Link AJ, Koga A, Vellek A, Hartl DL (1991) *He-T* family DNA sequences in the Y chromosome of *Drosophila melanogaster* share homology with the X-linked *Stellate* genes. Chromosoma 100: 118-124

Danilevskaya O, Lofsky A, Kurenova E, Pardue ML (1993) The Y chromosome of *Drosophila melanogaster* contains a distinctive subclass of *Het-A* related repeats. Genetics 134: 531-543

Das CC, Kaufman BP, Gay H (1964) Histone-protein transition in *Drosophila melanogaster*. Exp Cell Res 35: 507-514

Davidson EH (1986) Gene activity in early development. Academic Press, Orlando

Dearsly AL, Johnson RM, Barrett P, Sommerville J (1985) Identification of a 60-kDa phosphoprotein that binds stored messenger RNA of *Xenopus* oocytes. Eur J Biochem 150: 95-103

de Loos F, Dijkhof R, Grond CJ, Hennig W (1984) Lampbrush chromosome loop-specificity of transcript morphology in spermatocyte nuclei of *Drosophila hydei*. EMBO J. 3: 2845-2849

den Dunnen JT, Bakker E, Klein Breteler EG, Pearson PL, van Ommen GJB (1987) Direct detection of more than 50% of the Duchenne muscular dystrophy mutations by field inversion gels. Nature 329: 640-642

den Dunnen JT, Grootscholten PM, Bakker E, Bonden LAJ, Ginjaar HB, Wapenaar MC, van Paassen HMB, van Broeckhoven C, Pearson PL, van Ommen GJB (1989) Topography of the Duchenne muscular dystrophy (DMD) gene: FIGE and cDNA analysis of 194 cases reveals 115 deletions and 13 duplications. Am J Hum Genet 45: 835-847

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucl Acid Res 12: 387-395

Dobzhansky Th (1935) The Y chromosome of *Drosophila pseudoobscura*. Genetics 20: 366-376

Dolfini S (1971) Karyotype polymorphism in a cell population of *Drosophila melanogaster* cultured *in vitro*. Chromosoma 33: 196-208

Doolittle RF (1986) Of URFs and ORFs. A primer on how to analyze derived amino acid sequences. Oxford University Press, Oxford

Doolittle RF (1990) Molecular evolution: computer analyis of protein and nucleic acid sequences. Methods in Enzymology, Vol 183, Academic Press, San Diego

Dumanski JP, Ruttlegde M, Datta S (1988) Rapid minipreparations of bacteriophage λ DNA. Nucleic Acids Res 16: 9044

Duttaroy A, McCarron, M, Sitaraman K, Doughty G, Chovnick A (1990) The relationship between *P* elements and male recombination in *Drosophila melanogaster*. Genetics 124: 317-329

Eberl DF, Duyf BJ, Hilliker AJ (1993) The role of heterochromatin in the expression of a heterochromatic gene, the *rolled* locus of *Drosophila melanogaster*. Genetics 134: 277-292

Eicher EM, Hutchison KW, Philips SJ, Tucker PK, Lee BK (1989) A repeated segment on the mouse Y chromosome is composed of retroviral-related, Y-enriched and Y-specific sequences. Genetics 122: 181-192

Emery AEH (1988) Duchenne muscular dystrophy. Oxford monographs on medical genetics 15, Oxford University Press, Oxford

Emmerich P, Loos P, Jauch A, Hopman AHN, Wiegant J, Higgins MJ, White BN, van der Ploeg M, Cremer C, Cremer T (1989) Double in situ hybridization in combination with digital image analysis: a new approach to study interphase chromosome topography. Exp Cell Res 181: 126-140

Endow SA, Atwood KC (1988) Magnification: gene amplification by an inducible system of sister chromatid exchange. Trends Genet. 4: 348-351

Erickson RP (1990) Post-meiotic gene expression. Trends Genet 6: 264-269

Evans JP, Palmiter RP (1991) Retrotransposition of a mouse L1 element. Proc Natl Acad Sci USA 88: 8792-8795

Finnegan DJ (1990) Transposable elements. Dros Inf Serv 68: 371-382

Foster GG, Maddern RH, Mills AT (1980) Genetic instability in mass-rearing colonies of a sex-linked translocation strain of *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) during a field trial of genetic control. Theor Appl Genet 58: 169-175

Foster GG, Whitten MJ, Konovalov C, Arnold JTA, Maffi G (1981) Autosomal genetic maps of the Australian sheep blowfly, *Lucilia cuprina dorsalis* R.-D. (Diptera: Calliphoridae), and possible correlations with the linkage maps of *Musca domestica* L. and *Drosophila melanogaster* Mg. Genet Res 37: 55-69

Frasch M, Saumweber H, Symmons P, Dequin R (1985) Chromatin-associated antigens with specific distribution in embryonal tissues and on polytene chromosomes of *Drosophila melanogaster*. Hoppe Seyler's Z Physik Chem 366: 115-116

Frischauf A-M, Lehrach H, Poustka A, Murray N (1983) Lambda replacement vector carrying polylinker sequences. J Mol Biol 170: 827-842

Fuller MT (1986) Genetic analysis of spermatogenesis in *Drosophila*: the role of the testis-specific β tubulin and interacting genes in cellular morphogenesis. In Gall JG (ed) Gametogenesis and the early embryo. Alan R Liss, New York, pp 19-42

Fuller MT, Regan CL, Hays TS, Green LL (1989) Interacting genes identify interacting proteins involved in microtubule function in *Drosophila*. Cell Motil Cytoskel 14: 128-135

Gall JG (1956) On the submicroscopic structure of chromosomes. Brookhaven Symp Biol 8: 17-32

Gatti M, Pimpinelli S, Santini G (1976) Characterization of *Drosophila* heterochromatin. I. Staining and decondensation with Hoechst 33258 and quinacrine. Chromosoma 57: 351-375

Gatti M, Pimpinelli S (1983) Cytological and genetic analysis of the *Y* chromosome of *Drosphila melanogaster* I. Organization of the fertility factors. Chromosoma 88: 349-373

Gatti M, Pimpinelli S (1992) Functional elements in *Drosophila melanogaster* heterochromatin. Annu Rev Genet 26: 239-275

Gehtmann RC (1988) Crossing-over in males of higher Diptera (Brachycera). J Heredity 79: 344-350

Gepner J, Hays T (1993) A fertility region on the *Y* chromosome of *Drosophila melanogaster* encodes the structural gene for a dynein microtubule motor. Proc Natl Acad Sci USA 90: 11132-11136

Geyer PK, Green MM, Corces VG (1988) Mutant gene phenotypes mediated by a *Drosophila melanogaster* retrotransposon require sequences homologous to mammalian enhancers Proc Natl Acad Sci USA 85 8593-8597

Geyer PK, Corces VG (1992) DNA position-specific repression of transcription by a *Drosophila* zinc finger protein Genes Dev 6 1865-1873

Gibbons IR, Gibbons BH, Mocz G, Asai DJ (1991) Multiple nucleotide-binding sites in the sequence of dynein β heavy chain Nature 352 640-643

Glatzer KH (1975) Visualization of gene transcription of spermatocytes of *Drosophila hydei* Chromosoma 53 371-379

Glatzer KH (1979) Lengths of transcribed rDNA repeating units in spermatocytes of *Drosophila hydei* only genes without an intervening sequence are expressed Chromosoma 75 161-175

Glatzer KH (1980) Regular substructures within homologous transcripts of spread *Drosophila* germ cells Exp Cell Res 125 519-523

Glatzer KH (1984) Preservation of nuclear RNP antigens in male germ cell development of *Drosophila hydei* Mol Gen Genet 196 236-243

Glatzer KH, Meyer GF (1981) Morphological aspects of the genetic activity in primary spermatocyte nuclei of *Drosophila hydei* Biol Cell 41 165-172

Glatzer KH, Kloetzel P-M (1985) Antigens of cytoplasmic RNP particles of *D melanogaster* can be localized on distinct Y chromosomal structures in spermatocytes of *D hydei* Dros Inf Serv 61 84

Glatzer KH, Kloetzel P-M (1986) Differential chromosomal distribution of ribonucleoprotein antigens in nuclei of *Drosophila* spermatocytes J Cell Biol 103: 2113-2119

Glatzer KH, Bünemann H (1987) Transcripts of repetitive Y chromosomal sequences are involved in specific RNP structures in primary spermatocytes of *Drosophila hydei* In Mohri H (ed) New Horizons in sperm cell research Japanese Scientific Society Press, Tokyo - Gordon and Breach Scientific Publishers New York pp 107-115

Goldstein LSB, Hardy RW, Lindsley DL (1982) Structural genes on the Y chromosome of *Drosophila melanogaster* Proc Natl Acad Sci USA 79 7405-7409

Gonczy P, Viswanathan S, DiNardo S (1992) Probing spermatogenesis in *Drosophila* with P-element enhancer detectors Development 114 89-98

Gonzalez C Casal J, Ripoll P (1989) Relationship between chromosome content and nuclear diameter in early spermatids of *Drosophila melanogaster* Genet Res 54 205-212

Gould-Somero M Holland L (1974) The timing of RNA synthesis for spermiogenesis in organ cultures of *Drosophila melanogaster* testis Wilhelm Roux's Arch Dev Biol 174. 133-148

Green MM (1988) Mobile DNA elements and spontaneous gene mutation In Lambert ME McDonald JF, Weinstein IB (eds) Eukaryotic transposable elements as mutagenic agents Banbury Report 30 Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp 41-50

Grimaldi DA (1990) A phylogenetic, revised classification of genera in the Drosophilidae (Diptera) Bull Am Nat Hist 197 1-139

Grond C (1984) Spermatogenesis of *Drosophila hydei* Ph D Thesis, Department of Molecular and Developmental Genetics, Catholic University Nijmegen

Grond CJ, Siegmund I Hennig W (1983) Visualization of a lampbrush loop-forming fertility gene in *Drosophila hydei* Chromosoma 88 50-56

Grond CJ, Rutten RGJ, Hennig W (1984) Ultrastructure of the Y chromosomal lampbrush loops in primary spermatocytes of *Drosophila hydei* Chromosoma 89 85-95

Hackstein JHP (1987) Spermatogenesis in *Drosophila* In Hennig W (ed) Spermatogenesis, genetic aspects Results and problems in cell differentiation, vol 15 Springer, Berlin Heidelberg New York, pp 61-116

Hackstein JHP (1991) Spermatogenesis in *Drosophila* A genetic approach to cellular and subcellular differentiation Eur J Cell Biol 56 151-169

Hackstein JHP Hennig W (1982) New mutants *D hydei* Dros Inf Serv 58 195-203

Hackstein JHP, Leoncini O, Beck H, Peelen G, Hennig W (1982) Genetic fine structure of the Y chromosome of *Drosophila hydei* Genetics 101 257-277

Hackstein JHP, Hennig W, Siegmund I (1987) Y chromosome-specific mutations induced by a giant transposon in *Drosophila hydei* Mol Gen Genet 207 455-465

Hackstein JHP, Beck H, Hochstenbach R, Kremer H, Zacharias H (1990) Spermatogenesis in *Drosophila hydei* a genetic survey Roux's Archiv Dev Biol 199 251–280

Hackstein JHP, Glatzer KH, Hulsebos TJM (1991) Genetic and cytogenetic analysis of the "*Th-Ps*" region of the Y chromosome of *Drosophila hydei* evidence for dual functions of the lampbrush loop-forming fertility genes? Mol Gen Genet 227 293–305

Hackstein JHP, Hochstenbach R, van Breugel FMA (1992) Constructing balancer chromosomes for genetic screens in Drosophila hydei Theor Appl Genet 83 821–826

Hagel EJ, Miller OL Jr (1991) Ultrastructural analysis of polytene chromatin of *Drosophila melanogaster* reveals clusters of tightly linked co-expressed genes Chromosoma 100 173–186

Halfer C (1981) Interstrain heterochromatin polymorphisms in *Drosophila melanogaster* Chromosoma 84 195–206

Handel MA (1987) Genetic control of spermatogenesis in mice In Hennig W (ed) Spermatogenesis, genetic aspects Results and problems in cell differentiation, vol 15 Springer, Berlin Heidelberg New York, pp 1–62

Hardy RW (1975) The influence of chromosome content on the size and shape of sperm heads in *Drosophila melanogaster* and the demonstration of chromosome loss during spermiogenesis Genetics 79 231 264

Hardy RW (1980) Crystal aggregates in the primary spermatocytes of XO males of *D melanogaster* Dros Inf Serv 55 54–55

Hardy RW Tokuyasu KT, Lindsley DL (1981) Analysis of spermatogenesis in *Drosophila melanogaster* bearing deletions for Y-chromosome fertility genes Chromosoma 83 593–617

Hardy RW, Lindsley DL, Livak KJ, Lewis B, Siversten AL, Joslyn GL Edwards J, Bonaccorsi S (1984) Cytogenetic analysis of a segment of the Y chromosome of *Drosophila melanogaster* Genetics 107 591–610

Hareven D, Zuckerman M Lifschytz E (1986) Origin and evolution of the transcribed repeated sequences on the Y chromosome lampbrush loops of *Drosophila hydei* Proc Natl Acad Sci USA 83 125–129

Hartl DL, Ajioka JW, Cai H, Lohe AR, Lozovskaya ER, Smoller DA, Duncan IW (1992) Towards a *Drosophila* genome map Trends Genet 8 70–75

Hauschteck-Jungen E, Hartl DL (1982) Defective histone transition during spermiogenesis in heterozygous segregation distorter males of *Drosophila melanogaster* Chromosoma 87 535–559

Hays TS, Deuring R, Robertson B, Prout M, Fuller MT (1989) Interacting proteins identified by genetic interactions a missense mutation in a α tubulin fails to complement alleles of the testis-specific β tubulin gene of *Drosophila melanogaster* Mol Cell Biol 9 875–884

Hazelrigg T, Fornili P Kaufman TC (1982) A cytogenetic analysis of X-ray induced male sterlles on the *Y* chromosome of *Drosophila melanogaster* Chromosoma 87 535–559

Heidmann O, Heidmann T (1991) Retrotransposition of a mouse IAP sequence tagged with an indicator gene Cell 64 159–170

Heitz E (1933) Cytologische Untersuchungen an Dipteren III Die somatische Heteropyknose bei *Drosophila melanogaster* und ihre genetische Bedeutung Z Zellforsch Mikrosk Anat 20 237–287

Henderson SA, Woodruff RC, Thompson JN Jr (1978) Spontaneous chromosome breakage at male meiosis associated with male recombination in *Drosophila melanogaster* Genetics 88 93–107

Hennig I (1978) Vergleichend-zytologische und -genetische Untersuchungen am Genom der Fruchtfliegen-Arten *Drosophila hydei neohydei* und *eohydei* (Diptera Drosophilidae) Entomol Germ 4 211–223

Hennig I (1982) Hybrid' X-Y translocation chromosomes of *Drosophila hydei* and *D neohydei* Chromosoma 86 491–508

Hennig W (1967) Untersuchungen zur Struktur und Funktion des Lampenbürsten-Y-Chromosoms in der Spermatogenese von *Drosophila* Chromosoma 22 294–357

Hennig W (1968) Ribonucleic acid synthesis of the Y chromosome of *Drosophila hydei* J Mol Biol 38 227–239

Hennig W (1972) Highly repetitive DNA sequences in the genome of *Drosophila hydei* I Preferential localization in the X chromosomal heterochromatin J Mol Biol 71 407–417

Hennig W (1985) Y chromosome function and spermatogenesis in *Drosophila hydei* Adv Genet 23 179–234

Hennig W (1986) Heterochromatin and germ line-restricted DNA In Hennig W (ed) Germ line-soma differentiation Results and problems in cell differentiation, vol 13 Springer, Berlin Heidelberg New York Tokyo, pp 175–192

Hennig W (1987a) The Y chromosomal lampbrush loops of *Drosophila*. In Hennig W (ed) Structure and function of eukaryotic chromosomes Results and Problems in Cell Differentiation, vol 14, Springer Verlag, Berlin, pp 133–146

Hennig W (1987b) Spermatogenesis in *Drosophila* In Malacinski G (ed) Primers in developmental biology Vol 3 Developmental genetics in animals and plants Macmillan, New York, pp 239–274

Hennig W (1990) The Y chromosome of *Drosophila* In Adolph KW (ed) Chromosomes eukaryotic, prokaryotic and viral CRC Press, Boca Raton, Florida, USA, pp 213–238

Hennig W (1992) Spermatogenesis as a model system Andrologia 24 21–26

Hennig W (1993) Conventional protein coding genes in the *Drosophila* Y chromosome is the puzzle of the fertility gene function solved? Proc Natl Acad Sci (USA) 90 10904–10906

Hennig W, Hennig I, Stein H (1970) Repeated sequences in the DNA of *Drosophila* and their localization in giant chromosomes Chromosoma 32 31–63

Hennig W, Hennig I, Leoncini O (1974a) Some observations on spermatogenesis of *D hydei* Dros Inf Serv 51 127

Hennig W, Meyer GF Hennig I, Leoncini O (1974b) Structure and function of the Y chromosome of *Drosophila hydei* Cold Spring Harbor Symp Quant Biol 38 673–683

Hennig W, Link B, Leoncini O (1975) The location of the nucleolus organizer regions in *Drosophila hydei* Chromosoma 51: 57–63

Hennig W, Vogt P, Jacob G Siegmund I (1982) Nucleolus organizer regions in *Drosophila* species of the *repleta* group Chromosoma 87 279–292

Hennig W Huijser P, Vogt P, Jackle H, Edstrom J-E (1983) Molecular cloning of microdissected lampbrush loop DNA sequences of *Drosophila hydei* EMBO J 2 1741–1746

Hennig W, Briand RC, Hackstein J, Hochstenbach R, Kremer H, Lankenau D-H, Lankenau S Miedema K Potgens A (1989) Y chromosomal fertility genes of *Drosophila* a new type of eukaryotic genes Genome 31 561–571

Hennig W Kremer H (1990) Spermatogenesis of *Drosophila hydei* Int Rev Cytol 123 129–175

Hess O (1965a) Strukturdifferenzierungen im Y Chromosom von *Drosophila hydei* und ihre Beziehungen zu Gen-Aktivitaten I Mutanten der Funktionsstrukturen Verhandl Deut Zool Anz Suppl 28 156–163

Hess O (1965b) Strukturdifferenzierungen im Y Chromosom von *Drosophila hydei* und ihre Beziehungen zu Gen-Aktivitaten III Sequenz und Lokalisation der Schleifenbildungsorte Chromosoma 16 222–248

Hess O (1967a) Complementation of genetic activity in translocated fragments of the Y chromosome in *Drosophila hydei* Genetics 56 283–295

Hess O (1967b) Morphologische Variabilitat der Chromosomalen Funktionsstrukturen in den Spermatocytenkernen von *Drosophila*-Arten Chromosoma 21 429–445

Hess O (1968) The function of the lampbrush loops formed by the Y chromosome of *Drosophila hydei* in spermatocyte nuclei Molec Gen Genet 103 58–71

Hess O (1970) Genetic function correlated with unfolding of lampbrush loops by the Y chromosome in spermatocytes of *Drosophila hydei* Molec Gen Genet 106 328–346

Hess O (1980) Lampbrush chromosomes In Ashburner M, Wright TRF (eds) The genetics and biology of *Drosophila*, vol 2d, Academic Press London New York San Francisco, pp 1–32

Hess O, Meyer GF (1963a) Chromosomal differentiations of the lampbrush type formed by the Y chromosome in *Drosophila hydei* and *Drosophila neohydei* J Cell Biol 16 527–539

Hess O, Meyer GF (1963b) Artspezifische funktionelle Differenzierungen des Y-Heterochromatins bei *Drosophila*-Arten der *D hydei*-Subgruppe Port Acta Biol Ser A VII 29–46

Hess O, Meyer GF (1968) Genetic activities of the Y chromosome in *Drosophila* during spermatogenesis Adv Genet 14 171–228

Heyting C, Dietrich AJJ, Moens PB, Dettmers RJ, Offenberg HH, Redeker EJW, Vink ACG (1989) Synaptonemal complex proteins. Genome 31: 81-87

Hinton CW (1983) Relations between factors controlling crossing-over and mutability in males of *Drosophila ananassae*. Genetics 104: 95-112

Hiraizumi Y (1971) Spontaneous recombination in *Drosophila melanogaster* males. Proc Natl Acad Sci USA 68: 268-270

Hohn B, Collins J (1980) A small cosmid vector for efficient cloning of large DNA fragments. Gene 11: 291-298

Houck MA, Clark JB, Petersen KR, Kidwell MG (1991) Possible horizontal transfer of *Drosophila* genes by the mite *Proctolaelaps regalis*. Science 253: 1125-1129

Huijser P (1987) Genomic organization of microdissected *Y*-chromosomal lampbrush loop DNA sequences of *Drosophila hydei*. Ph.D. Thesis, Department of Molecular and Developmental Genetics, Catholic University of Nijmegen

Huijser P, Hennig W (1987) Ribosomal DNA-related sequences in a Y chromosomal lampbrush loop of *Drosophila hydei*. Mol Gen Genet 206: 441-451

Huijser P, Hennig W, Dijkhof R (1987) Poly(dC-dA)/(dG-dT) repeats in the *Drosophila* genome: a key function for dosage compensation and position effects? Chromosoma 95: 209-215

Huijser P, Kirchhoff C, Lankenau D-H, Hennig W (1988) Retrotransposon-like sequences are expressed in Y chromosomal lampbrush loops of *Drosophila hydei*. J Mol Biol 203: 689-697

Huijser P, Beckers L, Top B, Hermans M, Sinke R, Hennig W (1990) Poly[d(C-A)]. poly[d(G-T)] is highly transcribed in the testis of *Drosophila hydei*. Chromosoma 100: 48-55

Hulsebos TJM, Hackstein JHP, Hennig W (1983) Involvement of *Y* chromosomal loci in the synthesis of *Drosophila hydei* sperm proteins. Dev Biol 100: 238-243

Hulsebos TJM, Hackstein JHP, Hennig W (1984) Lampbrush loop-specific protein of *Drosophila hydei*. Proc Natl Acad Sci USA 81: 3404-3408

Ingham PW (1988) The molecular genetics of embryonic pattern formation in *Drosophila*. Nature 335: 25-34

Ingman-Baker J, Candido EPM (1980) Proteins of the *Drosophila melanogaster* reproductive system: two-dimensional gel patterns of proteins synthesized in the XO, XY, and XYY testis and paragonial gland and evidence that the Y chromosome does not code for structual sperm proteins. Biochem. Genet. 18: 809-828

Irvine KD, Helfand SL, Hogness DS (1991) The large upstream control region of the *Drosophila* homeotic gene *Ultrabithorax*. Development 111: 407-424

Jamieson BGM (1987) The ultrastructure and phylogeny of insect spermatozoa. Cambridge University Press, Cambridge

Jarrell KA, Meselson M (1991) *Drosphila* retrotransposon promoter includes an essential sequence at the initiation site and requires a downstream promoter for full activity Proc Natl Acad Sci USA 88: 102-104

Jensen S, Heidmann T (1991) An indicator gene for detection of germline retrotransposition in transgenic *Drosophila* demonstrates RNA-mediated transposition of the LINE *I* element. EMBO J 10: 1927-1937

John B (1988) The biology of heterochromatin. In Verma RS (ed) Heterochromatin: molecular and structural aspects. Cambridge University Press, Cambridge, pp1-147

Jokusch H (1966) Relations between temperature-sensitivity, amino-acid replacement and quarternary structure of mutant protein. Biochem Biophys Res Commun 24: 577-583

Junakovic N, Di Franco C, Best-Pelhomme M, Echalier G (1988) On the transposition of copia-like nomadic elements in cultured *Drosophila* cells. Chromosoma 97: 212-218

Kaltschmidt B, Glätzer KH, Michiels F, Leiss D, Renkawitz-Pohl R (1991) During *Drosophila* spermatogenesis β_1, β_2 and β_3 tubulin isotypes are cell-type specifically expressed but nevertheless have the potential to coassemble in the axoneme of the transgenic flies. Eur J Cell Biol 54: 110-120

Karn J, Brenner S, Barnett L, Cesareni G (1980) Novel bacteriophage λ cloning vector. Proc Natl Acad Sci USA 77: 5172-5176

Kemphues KJ, Raff RA, Kaufman TC, Raff EC (1979) Mutation in a structural gene for a β tubulin specific to testis in *Drosophila melanogaster*. Proc Natl Acad Sci USA 76: 3991-3995

Kemphues KJ, Raff EC, Raff RA, Kaufman TC (1980) Mutation in a testis-specific β tubulin in *Drosophila*: analysis of its effects on meiosis and map location of the gene. Cell 21: 445–451

Kemphues KJ, Kaufman TC, Raff RA, Raff EC (1982) The testis-specific β tubulin subunit in *Drosophila melanogaster* has multiple functions in spermatogenesis. Cell 31: 655–670

Kennison JA (1981) The genetic and cytological organization of the Y chromosome of *Drosophila melanogaster*. Genetics 98: 529–548

Kennison JA (1983) Analysis of Y-linked mutations to male sterility in *Drosophila melanogaster*. Genetics 103: 219–234

Kidwell MG (1993) Lateral transfer in natural populations of eukaryotes. Annu Rev Genet 27: 235–256

Kidwell MG, Kidwell JF, Sved JA (1977) Hybrid dysgenesis in *Drosophila melanogaster*: a syndrome of aberrant traits including mutation, sterility and male recombination. Genetics 86: 813–833

Kiefer BI (1966) Ultrastructural abnormalities in developing sperm of X/0 *Drosophila melanogaster*. Genetics 54: 1441–1452

Kiefer BI (1968) Y-mutants and spermiogenesis in *Drosophila melanogaster*. Genetics 60: 192

Kiefer BI (1969) Phenotypic effects of Y chromosome mutations in *Drosophila melanogaster*. I. Spermiogenesis and sterility in *kl-1*$^-$ males. Genetics 61: 157–166

Kiefer BI (1973) Genetics of sperm development in *Drosophila*. In: Ruddle FH (ed) General mechanisms of development. Academic Press, New York London San Francisco, pp 47–102

King RC, Burnett RG (1959) Autoradiographic study of uptake of tritiated glycine, thymidine, and uridine by fruitfly ovaries. Science 129: 1674–1675

Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM (1987) Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell 50: 509–517

Koopmans-Frankel AW, Peters U, Meyer GF (1971) Variation in fertility of two wild type strains of *Drosophila melanogaster* Meigen. Chromosoma 34: 113–128

Kremer H, Hennig W, Dijkhof R (1986) Chromatin organization in the male germ line of *Drosophila hydei*. Chromosoma 94: 147–161

Kuff EL, Lueders KK (1988) The intracisternal A-particle gene family: structure and functional aspects. Adv Cancer Res 51: 183–276

Kuhn R, Schafer U, Schafer M (1988) *Cis*-acting regions sufficient for spermatocyte-specific transcriptional and spermatid-specific translational control of the *Drosophila melanogaster* gene *mst(3)gl-9*. EMBO J 7: 447–454

Kunz W, Petersen G, Renkawitz-Pohl R, Glatzer KH, Schafer M (1981) Distribution of spacer length classes and the intervening sequence among different nucleolus organizers in *Drosophila hydei*. Chromosoma 83: 145–158

Kurek R (1991) Versuche zur Charakterisierung der Y chromosomalen Fertilitatsgene von *Drosophila hydei* durch YAC-Klonierung. Diplomarbeit, Mathematisch-Naturwissenschaftliche Fakultat, Heinrich Heine Universitat, Düsseldorf

Lachaume P, Bouhidel K, Mesure M, Pinon H (1992) Spatial and temporal expression of the I factor during oogenesis in *Drosophila melanogaster*. Development 115: 729–735

Laird CD, Wilkinson LE, Foe VE, Chooi WY (1976) Analysis of chromatin-associated fiber arrays. Chromosoma 58: 169–192

Lankenau D-H (1990) Molecular structure and evolution of a retrotransposon family in *Drosophila*. Ph D Thesis, Department of Molecular and Developmental Genetics, Catholic University of Nijmegen

Lankenau D-H (1993) The retrotransposon family *micropia* in *Drosophila* species. In: McDonald JF (ed) Transposable elements and evolution. Kluwer Academic Publishers, Amsterdam, pp 232–241

Lankenau D-H, Huijser P, Jansen E, Miedema K, Hennig W (1988) *Micropia*: a retrotransposon of *Drosophila* combining structural features of DNA viruses, retroviruses and non-viral transposable elements. J Mol Biol 204: 233–246

Lankenau D-H, Huijser P, Hennig W (1989) Characterization of the long terminal repeats of *micropia* elements microdissected from the Y-chromosomal lampbrush loops "*Threads*" of *Drosophila hydei*. J Mol Biol 209: 493–497

Lankenau D-H, Huijser P, Jansen E, Miedema K, Hennig W (1990) DNA sequence comparison of *micropia* transposable elements from *Drosophila hydei* and *Drosophila melanogaster*. Chromosoma 99: 111-117

Lankenau S, Corces VG, Lankenau D-H (1994) The *Drosophila micropia* retrotransposon encodes a testis-specific antisense RNA complementary to reverse transcriptase. Mol Cell Biol 14, in press

Lawrence JB, Villnave CA, Singer RH (1988) Sensitive, high-resolution chromatin and chromosome mapping in situ: presence and orientation of two closely integrated copies of EBV in a lymphoma cell line. Cell 52: 51-61

Lawrence JB, Singer RH, McNeil JA (1990) Interphase and metaphase resolution of different distances within the human dystrophin gene. Science 249: 928-932

Leoncini O (1977) Temperatursensitive Mutanten im Y Chromosom von *Drosphila hydei*. Chromosoma 63: 329-357

Lester DS, Crozier RH, Shipp E (1979) Recombination in the male housefly, *Musca domestica*. Experientia 35: 175-176

Lifschytz E (1974) Genes controlling chromosome activity. An X-linked mutation affecting Y-lampbrush loop activity in *Drosophila hydei* Chromosoma 47: 415-427

Lifschytz E (1975) Genes controlling chromosome activity: the role of genes blocking Y-lampbrush loop propagation. Chromosoma 53: 231-241

Lifschytz E (1979) A procedure for the cloning and identification of Y-specific middle repetitive sequences in *Drosophila hydei*. J Mol Biol 133: 267-277

Lifschytz E (1987) The developmental program of spermiogenesis in *Drosophila*: a genetic analysis. Int Rev Cytol 109: 211-258

Lifschytz E, Hareven D (1977) Gene expression and the control of spermatid morphogenesis in *Drosophila melanogaster*. Dev Biol 58: 276-294

Lifschytz E, Meyer GF (1977) Characterization of male meiotic sterile mutations in *Drosophila melanogaster*. The genetic control of meiotic divisions and gametogenesis. Chromosoma 64: 371-392

Lifschytz E, Hareven D, Azriel A, Brodsly H (1983) DNA clones and RNA transcripts of four lampbrush loops from the Y chromosome of *Drosophila hydei*. Cell 32: 191-199

Lifschytz E, Hareven D (1985) Molecular evidence for partial inactivation of Y loops in T(X;Y)56 males from *D. hydei*. Molec Gen Genet 199: 46-52

Lindsley DL, Grell EH (1969) Spermiogenesis without chromosomes in *Drosophila melanogaster*. Genetics 61, Suppl 1: 69-78

Lindsley DL, Lifschytz E (1972) The genetic control of spermatogenesis in *Drosophila*. In Beatty RA, Gluecksohn-Waelsch S (eds) The genetics of the spermatozoon. Bogtrykkerlet Forum, Copenhagen, pp 203-222

Lindsley DL, Sandler L, Baker BS, Carpenter ATC, Denell RE, Hall JC, Jacobs PA, Miklos GLG, Davis BK, Gethmann RC, Hardy RW, Hessler A, Miller SM, Nozawa H, Parry DM, Gould-Somero M (1972) Segmental aneuploidy and the genetic gross structure of the *Drosophila* genome. Genetics 71: 157-184

Lindsley DL, Tokuyasu KT (1980) Spermatogenesis. In Ashburner M, Wright TRF (eds) The genetics and biology of *Drosophila*, vol 2d, Academic Press, London New York San Francisco, pp 225-294

Lindsley DL, Zimm G (1987) The genome of *Drosophila melanogaster*. Part 3: rearrangements. Dros Inf Serv 65

Livak KJ (1984) Organization and mapping of a sequence on the *Drosophila melanogaster* X and Y chromosomes that is transcribed during spermatogenesis. Genetics 107: 611-634

Livak KJ (1990) Detailed structure of the *Drosophila melanogaster Stellate* genes and their transcripts. Genetics 124: 303-316

Lohe AR, Brutlag DL (1986) Multiplicity of satellite DNA sequences in *Drosophila melanogaster*. Proc Natl Acad Sci USA 83: 696-700

Lohe AR, Brutlag DL (1987) Identical satellite DNA sequences in sibling species of *Drosophila*. J Mol Biol 194: 161-170

Lohe A, Roberts P (1988) Evolution of satellite DNA sequences in *Drosophila*. In: Verma RS (ed) Heterochromatin: molecular and structural aspects. Cambridge University Press, Cambridge, pp 148-186

Macdonald PM (1992) The *Drosophila pumilio* gene: an unusually long transcription unit and an unusual protein. Development 114: 221-232

Mahowald AP (1992) Germ plasm revisited and illuminated. Science 255: 1216–1217
Mainx (1964) The genetics of *Megaselia scalaris* Loew (Phoridae): a new type of sex determination in Diptera. Am Nat 98: 415–430
Manley JL (1988) Polyadenylation of mRNA precursors. Bioch Bioph Acta 950: 1–12
Manuelidis L, Borden J (1988) Reproducible compartmentalization of individual chromosome domains in human CNS cells revealed by in situ hybridization and three-dimensional reconstruction. Chromosoma 96: 397–410
Marlor RL, Parkhurst SM, Corces VG (1986) The *Drosophila melanogaster gypsy* transposable element encodes putative gene products homologous to retroviral proteins. Mol Cell Biol 6: 1129–1134
Martin G, Wiernasz D, Schedl P (1983) Evolution of *Drosophila* repetitive-dispersed DNA. J Mol Evol 19: 203–213
Marsh JL, Wieschaus E (1978) Is sex determination in germ line and soma controlled by separate genetic mechanisms? Nature 272: 249–251
Maruyama K, Hartl DL (1991a) Evolution of the transposable element *mariner* in *Drosophila* species. Genetics 128: 319–329
Maruyama K, Hartl DL (1991b) Evidence for interspecific transfer of the transposable element mariner between *Drosophila* and *Zaprionus*. J Mol Evol 33: 514–524
Matsuda M, Imai HT, Tobari YN (1983) Cytogenetic analysis of recombination in males of *Drosophila ananassae*. Chromosoma: 88: 286–292
Matthews KA, Miller D, Kaufman TC (1989) Developmental distribution of RNA and protein products of the *Drosophila* α tubulin gene family. Dev Biol 132: 45–61
Mazo AM, Mizrokhi LJ, Karavanov AA, Sedkov YA, Krichevskaja AA, Ilyin YV (1989) Suppression in *Drosophila*: *su(Hw)* and *su(f)* gene products interact with a region of *gypsy (mdg4)* regulating its transcriptional activity. EMBO J 8: 903–911
McCloskey JD (1966) The problem of gene activity in the sperm of *Drosophila melanogaster*. Am Natur 100: 211–218
McDonald JF (1990) Macroevolution and retroviral elements. BioScience 40: 183–191
Mcgregor HC, Callan HG (1962) The actions of enzymes on lampbrush chromosomes. Quart J Microsc Sci 103: 173–203
McLean C, Bucheton A, Finnegan DJ (1993) The 5' untranslated region of the *I* factor, a long interspersed nuclear element-like retrotransposon of *Drosophila melanogaster*, contains an internal promoter and sequences that regulate transcription. Mol Cell Biol 13: 1042–1050
Meizer S, Glätzer KH (1985) Localization of RNP antigens in primary spermatocytes of *Drosophila melanogaster* by indirect immunofluorescence and their correlation to fertility factors. Dros Inf Serv 61: 121
Meyer GF (1960) The fine structure of spermatocyte nuclei of *Drosophila melanogaster*. In Houwink AL, Spit BJ (eds) Proc Eur Reg Conf Electron Microscopy Delft 1960, Nederlandse Vereniging voor Electronenmicroscopie, Delft, pp 951–954
Meyer GF (1963) Die Funktionsstrukturen des *Y*-Chromosoms in den Spermatocytenkernen von *Drosophila hydei*, *D. neohydei*, *D. repleta* und einigen anderen *Drosophila*-Arten. Chromosoma 14: 207–255
Meyer GF (1964) Die parakristallinen Körper in den Spermienschwänzen von *Drosophila*. Z Zellforsch Mikrosk Anat 62: 762–784
Meyer GF (1965) A reliable cytological method for classification of *Drosophila* species. Dros Inf Serv 40: 80
Meyer GF(1968) Spermiogenese in normalen und Y-defizienten Mänchenn von *Drosophila melanogaster* und *D. hydei*. Z Zellforsch Mikrosk Anat 84: 141–175
Meyer GF (1969) Experimental studies on spermatogenesis in *Drosophila*. Genetics 61, Suppl 1: 79–92
Meyer GF (1972) Influence of the Y chromosome on fertility and phenotype of *Drosophila* spermatozoa. In Beatty RA, Gluecksohn-Waelsch S (eds) The genetics of the spermatozoon. Bogtrykkeriet Forum, Copenhagen, pp 387–405
Meyer GF, Hess O, Beermann W (1961) Phasenspezifische Funktionsstrukturen in Spermatocytenkernen von *Drosophila melanogaster* und ihre Abhängigkeit vom Y-Chromosom. Chromosoma 12: 676–716
Meyer GF, Hess O (1965) Strukturdifferenzierungen im Y Chromosom von *Drosophila hydei* und ihre Beziehungen zu Gen-Aktivitäten. II. Effekt der RNS-Synthese-Hemmung durch Actinomycin. Chromosoma 16: 249–270
Meyer GF Hennig W (1974) The nucleolus in primary spermatocytes of *Drosophila hydei*. Chromosoma 46: 121–144

Meyerowitz EM, Hogness DS (1982) Molecular organization of a *Drosophila* puff site that responds to ecdysone Cell 28 165-176

Michiels F, Falkenburg D, Müller AM, Hinz U, Otto U, Bellmann R, Glätzer KH, Brand R, Bialojan S, Renkawitz-Pohl R (1987) Testis-specific β_2 tubulins are identical in *Drosophila melanogaster* and *D hydei* but differ from the ubiquitous β_1 tubulin Chromosoma 95 387-395

Michiels F, Gasch A, Kaltschmidt B, Renkawitz-Pohl R (1989) A 14 bp promoter element directs the testis-specificity of the *Drosophila* β_2 tubulin gene EMBO J 8 1559-1565

Miedema K (1994) Muscle myosin heavy chain gene expression in the testis of *Drosophila hydei* and *Drosophila melanogaster* Ph D Thesis, Department of Molecular and Developmental Genetics, Catholic University of Nijmegen

Miklos GLG (1982) Sequencing and manipulating highly repeated DNA In Dover GA, Flavell, RB (eds) Genome evolution Academic Press, London, New York, pp 41-68

Miklos GLG (1985) Localized highly repetitive DNA sequences in vertebrate and invertebrate genomes In MacIntyre RJ (ed) Molecular evolutionary genetics Plenum Press, New York, pp 241-321

Miklos GLG, Nankivell RN (1976) Telomeric satellite DNA functions in regulating recombination Chromosoma 56 1430-167

Miklos GLG, Cotsell JN (1990) Chromosome structure at interfaces between major chromatin types alpha and beta-heterochromatin BioEssays 12 1-6

Milani R (1975) The house fly *Musca domestica* In King RC (ed) Handbook of genetics, vol 3 Invertebrates of genetic interest Plenum Press, New York, pp 377-399

Miller DD, Stone L (1962) A reinvestigation of karyotype in *Drosophila affinis* and related species J Heredity 53 12-24

Miller DD, Roy R (1964) Further data on the Y chromosome types in *Drosophila athabasca* Can J Genet Cytol 6 334-348

Miller OL, Beatty BR (1969) Visualization of nucleolar genes Science 164 955-957

Mischke D, Pardue ML (1982) Organization and expression of α tubulin genes in *Drosophila melanogaster* J Mol Biol 156 449-466

Mizrokhi LJ, Mazo AM (1990) Evidence for horizontal transmission of the mobile element *jockey* between distant *Drosophila* species Proc Natl Acad Sci USA 87 9216-9220

Mizrokhi LJ, Mazo AM (1991) Cloning and analysis of the mobile element *gypsy* from *D virilis* Nucl Acid Res 19 913-916

Montgomery EA, Charlesworth B, Langley CH (1987) A test for the role of natural selection in the stabilization of transposable element copy number in a population of *Drosophila melanogaster* Genet Res 49 31-41

Montgomery EA, Huang S-M, Langley CH, Judd BH (1991) Chromosome rearrangement by ectopic recombination in *Drosophila melanogaster* genome structure and evolution Genetics 129 1085-1098

Morgan, TH (1912) Complete linkage in the second chromosome of the male of *Drosophila* Science 36 718-720

Morgan, TH (1914) No crossing-over in the male of *Drosophila* of genes in the second and third pairs of chromosomes Biol Bull (Woods Hole) 26 195-204

Moser H (1984) Duchenne muscular dystrophy pathogenetic aspects and genetic prevention Hum Genet 66 17-40

Muller HJ (1918) Genetic variability, twin hybrids and constant hybrids, in a case of balanced lethal factors Genetics 3 422-449

Neesen J, Bünemann H, Heinlein UAO (1993) The *Drosophila hydei* gene Dhmst101(1) encodes a testis-specific, repetitive, axoneme-associated protein with differential abundance in Y chromosomal deletion mutant flies Devel Biol, in press

Neuhaus ME (1939) A cytogenetic study of the Y chromosome in *Drosophila melanogaster* J Genetics 37 229-254

Nicoletti B and Lindsley DL (1960) Translocations between the X and Y chromosomes of *Drosophila melanogaster* Genetics 45 1705-1722

Nissani M, Brink NG, Nissani HG (1978) The site of function of the Y chromosome in *Drosophila melanogaster* Mol Gen Genet 165 221-224

Offenberg HH, Dietrich AJJ, Heyting C (1991) Tissue distribution of two major components of synaptonemal complexes of the rat Chromosoma 101 83-91

Ogawa K (1991) Four ATP-binding sites in the midregion of the β heavy chain of dynein. Nature 352: 643-645

Ohno S (1967) Sex chromosomes and sex linked genes. Springer-Verlag, Berlin Heidelberg New York

Olivieri G, Olivieri A (1965) Autoradiographic study of nucleic acid synthesis during spermatogenesis in *Drosophila melanogaster*. Mutation Res 2: 366-380

Papenbrock T (1991) Molekulare Analyse der Riesentranskripte aus den *Y* chromosomalen Lampenbürstenschleifen von *Drosophila hydei*. Ph.D. Thesis, Institut für Genetik, Heinrich Heine Universität, Düsseldorf

Pardue ML, Bendena WG, Garbe JC (1987) Heat shock: puffs and response to environmental stress. In Hennig W (ed) Structure and function of eukaryotic chromosomes. Results and problems in cell differentiation, vol 14. Springer, Berlin Heidelberg New York, pp 121-131

Pardue ML, Hennig W (1990) Heterochromatin: junk or collectors item? Chromosoma 100: 3-7

Parkhurst SM, Corces VG (1986) Interactions among the *gypsy* transposable element and the *yellow* and the *suppressor of Hairy-wing* loci in *Drosophila melanogaster*. Mol Cell Biol 6: 47-53

Parkhurst SM, Corces VG (1987) Developmental expression of *Drosophila melanogaster* retrovirus-like transposable elements. EMBO J 6: 419-424

Parkhurst SM, Harrison DA, Remington MP, Spana C, Kelley RL, Coyne RS, Corces VG (1988) The *Drosophila su(Hw)* gene, which controls the phenotypic effect of the *gypsy* transposable element, encodes a putative DNA-binding protein. Genes Dev 2: 1205-1215

Passos-Bueno MR, Bakker E, Kneppers ALJ, Takata RI, Rapaport D, den Dunnen JT, Zatz M, van Ommen GJB (1992) Different mosaicism frequencies for proximal and distal Duchenne muscular dystrophy (DMD) mutations indicate difference in etiology and recurrence risk. Am J Hum Genet 51: 1150-1155

Patterson JT, Stone WS (1952) Evolution in the genus *Drosophila*. Macmillan, New York

Peacock WJ, Brutlag D, Goldring E, Appels R, Hinton CW, Lindsley DL (1973) The organization of highly repeated DNA sequences in *Drosophila melanogaster* chromosomes. Cold Spring Harbor Symp Quant Biol 38: 405-416

Peacock WJ, Lohe AR, Gerlach WL, Dunsmuir P, Dennis ES, Appels R (1977) Fine structure and evolution of DNA in heterochromatin. Cold Spring Harbor Symp Quant Biol 42: 1121-1135

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85: 244-2448

Peifer M, Bender W (1988) Sequences of the *gypsy* transposon of *Drosophila* necessary for its effects on adjacent genes. Proc Natl Acad Sci USA 85: 9650-9654

Pélisson A, Finnegan DJ, Bucheton A (1991) Evidence for retrotransposition of the *I* factor, a LINE element of *Drosophila melanogaster*. Proc Natl Acad Sci USA 88: 4907-4910

Penrad-Mobayed M, Sourrouille P, Bonnanfant-Jais ML, N'Da E, Edström JE, Angelier N (1991) Microdissection and cloning of DNA from landmark loops of amphibian lampbrush chromosomes. Chromosoma 101: 180-188

Perje A-M (1948) Studies on the spermatogenesis in *Musca domestica*. Hereditas 34: 209-232

Philips SJ, Birkenmeier EH, Callahan R, Eicher EM (1982) Male and female mouse DNAs can be discriminated using retroviral probes. Nature 297: 241-243

Pimpinelli S, Santini G, Gatti M (1976) Characterization of *Drosophila* heterochromatin. II. C- and N- banding. Chromosoma 52: 377-386

Pimpinelli S, Santini G, Gatti M (1978) ^{3}H-Actinomycin-D binding to mitotic chromosomes of *Drosophila melanogaster*. Chromosoma 66: 389-395

Pinol-Roma S, Swanson MS, Gall JG, Dreyfuss G (1989) A novel heterogeneous nuclear RNP protein with a unique distribution on nascent transcripts. J Cell Biol 109: 2575-2587

Pisano C, Bonaccorsi S, Gatti M (1993) The *kl-3* loop of the Y chromosome of *Drosophila melanogaster* binds a tektin-like protein. Genetics 133: 569-579

Plasterk RHA (1993) Molecular mechanisms of transposition and its control. Cell 74: 781-786

Proudfoot NJ (1989) How RNA polymerase II terminates transcription in higher eukaryotes Trends Biochem Sci 14 105-110

Porter ME, Johnson KA (1989) Dynein structure and function Ann Rev Cell Biol 5 119-151

Raff EC, Fuller MT (1984) Genetic analysis of microtubule function in *Drosophila* In Borisy G, Cleveland D, Murphy D (eds) Molecular biology of the cytoskeleton Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Rasmussen SW (1973) Ultrastructural studies of spermatogenesis in *Drosophila melanogaster* Z Zellforsch Mikrosk Anat 140 125-144

Rasmussen SW (1974) Studies on the development and ultrastructure of the synaptonemal complex in *Drosophila melanogaster* CR Trav Carlsberg 39 443-468

Regan CL, Fuller MT (1988) Interacting genes that affect microtubule function the *nc2* allele of the *haywire* locus fails to complement mutations in the testis-specific β tubulin gene of *Drosophila* Genes Dev 2 82-92

Regan CL, Fuller MT (1990) Interacting genes that affect microtubule function in *Drosophila melanogaster* two classes of mutations revert the failure to complement between hay^{nc2} and mutations in tubulin genes Genetics 125 77-90

Renkawitz R (1978a) Characterization of two moderately repetitive DNA components localized within the β-heterochromatin of *Drosophila hydei* Chromosoma 66 225-236

Renkawitz R (1978b) Two highly repetitive DNA satellites of *Drosophila hydei* localized within the α-heterochromatin of specific chromosomes Chromosoma 66 237-248

Renkawitz-Pohl R, Glatzer KH, Kunz W (1980) Characterization of cloned ribosomal DNA of *Drosophila hydei* Nucleic Acids Res 8 4593-4611

Rice WR (1987) Genetic hitchhiking and the evolution of reduced genetic activity of the Y sex chromosome Genetics 116 161-167

Risau W, Symmons P, Saumweber H, Frasch M (1983) Nonpackaging and packaging proteins of hnRNA in *Drosophila melanogaster* Cell 33 529-541

Ritossa FM (1976) The *bobbed* locus In Ashburner M, Novitski E (eds) The genetics and biology of *Drosophila* vol 1b, Academic Press, New York, pp 801-846

Robinson AS (1989) Genetic sexing methods in the mediterranean fruit fly, *Ceratitis capitata* In Robinson AS, Hooper G (eds) Fruit flies Their biology, natural enemies and control World crop pests, vol 3, Elsevier, Amsterdam Oxford New York Tokyo, pp 57-67

Roseman RR Pirrotta V, Geyer PK (1993) The *su(Hw)* protein insulates expression of the *Drosophila melanogaster white* gene from chromosomal position effects EMBO J 12 435-442

Roth MB, Gall JG (1987) Monoclonal antibodies that recognize transcription unit proteins on newt lampbrush chromosomes J Cell Biol 105 1047-1054

Rückert J (1892) Zur Entwicklungsgeschichte des Ovarialeies bei Selachiern Anat Anz 7 107-158

Russell SRH, Kaiser K (1993) *Drosophila melanogaster* male germ line-specific transcripts with autosomal and Y-linked genes Genetics 134 293-308

Rykowski MC, Parmelee SJ, Agard DA, Sedat JW (1988) Precise determination of the molecular limits of a polytene chromosome band regulatory sequences for the *Notch* gene are in the interband Cell 54 461-472

Saigo K, Kugimiya W, Matsuo Y, Inouye S, Yoshioka K, Yuki S (1984) Identification of the coding sequence for a reverse transcriptase-like enzyme in a transposable genetic element in *Drosophila melanogaster* Nature 312 659-661

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning A laboratory manual Second Edition Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sandmeyer SB, Hansen LJ, Chalker DL (1990) Integration specificity of retrotransposons and retroviruses Annu Rev Genet 24 491-518

Saumweber H, Symmons P, Kabisch R, Will H, Bonhoeffer F (1980) Monoclonal antibodies against chromosomal proteins of *Drosophila melanogaster* Chromosoma 80 253-275

Schafer M, Kuhn R, Bosse F, Schafer U (1990) A conserved element in the leader mediates post-meiotic translation as well as cytoplasmic polyadenylation of a *Drosophila* spermatocyte mRNA EMBO J 9 4519-4525

Schafer M, Borsch D, Hülster A, Schäfer U (1993) Expression of a gene duplication ecoding conserved sperm tail proteins is translationally regulated in *Drosophila melanogaster* Molec Cell Biol 13 1708–1718

Schäfer U (1978) Sterility in *Drosophila hydei* x *D neohydei* hybrids Genetica 49 205–214

Schafer U, Kunz W (1975) Two separated nucleolus organizers on the *Drosophila hydei* Y chromosome Molec Gen Genet 137 365–368

Schäfer U, Nahmias J (1985) Hybrid dysgenesis-induced Y chromosomal sterility in *Drosophila melanogaster* Mol Gen genet 200 182–184

Schmekel K, Wahrman J, Daneholt B (1993) Solitary and synaptonemal complex-associated recombination nodules in pro-nurse cells during oogenesis in *Drosophila melanogaster* Chromosoma 102 396–402

Schuldt C, Kloetzel P-M (1985) Analysis of cytoplasmic 19 S ring-type particles in *Drosophila* which contain hsp23 at normal growth temperature Dev Biol 110 65–74

Sheimoen AW, O'Farrell PH (1991) Progression of the cell cycle through mitosis leads to abortion of nascent transcripts Cell 67 303–310

Simmons GM (1992) Horizontal transfer of hobo transposable elements within the *Drosophila melanogaster* species complex evidence from DNA sequencing Mol Biol Evol 9 1050–1060

Smith KD, Young, KE, Talbot CC Jr, Schmeckpeper BJ (1987) Repeated DNA of the human Y chromosome Development 101 (Suppl) 77–92

Smith PA, Corces VG (1992) The suppressor of Hairy-wing binding region is required for gypsy mutagenesis Mol Gen Genet 233 65–70

Smoller DA, Petrov D, Hartl DL (1991) Characterization of bacteriophage P1 library containing inserts of *Drosophila* DNA of 75–100 kilobase pairs Chromosoma 100 487–494

Sneddon A, Flavell AJ (1989) The transcriptional control regions of the *copia* retrotransposon Nucl Acids Res 17 4025–4035

Southern EM (1970) Base sequence and evolution of guinea-pig α-satellite DNA Nature 227 794–798

Spana C, Harrison DA, Corces VG (1988) The *Drosophila melanogaster suppressor of Hairy-wing* protein binds to specific sequences of the *gypsy* retrotransposon Genes Dev 2 1414–1423

Spicer GS (1988) Molecular evolution among some *Drosophila* species groups as indicated by two-dimensional electrophoresis J Mol Evol 27 250–260

Springer R (1958) Die Koppelungsverhältnisse bei den beiden Geschlechtern von *Megaselia scalaris* Loew (= *Aphiochaeta xanthina* Speis) (Phoridae) Z Vererbungslehre 89 373–376

Stacey SN, Lanham RA, Brock HW, Grigliatti TA (1986) Distribution and conservation of mobile elements in the genus *Drosophila* Mol Biol Evol 3 522–534

Stalker HD (1945) On the biology and genetics of *Scaptomyza gramium* Fallen (Diptera, Drosophilidae) Genetics 30 266–279

Steinemann M, Steinemann S (1991) Preferential Y chromosomal localization of *TRIM*, a novel transposable element of *Drosophila miranda obscura* group Chromosoma 101 169–179

Steinemann M, Steinemann S (1992) Degenerating Y chromosome of *Drosophila miranda* a trap for retrotransposons Proc Natl Acad Sci USA 89 7591–7595

Steinemann M, Steinemann S, Lottspeich F (1993) How Y chromosomes become genetically inert Proc Natl Acad Sci USA 90 5737–5741

Steinmann-Zwicky M, Schmid H, Nothiger R (1989) Cell-autonomous and inductive signals can determine the sex of the germ line of *Drosophila* by regulating the gene *Sxl* Cell 57 157–166

Stern C (1929) Untersuchungen über Aberrationen des Y Chromosoms von *Drosophila melanogaster* Z Indukt Abstammungs- Vererbungsl 51 253–353

Sternberg NL (1992) Cloning high molecular weight DNA fragments by the bacteriophage P1 system Trends Genet 8 11–16

St Johston D, Nüsslein-Volhard C (1992) The origin of pattern and polarity in the *Drosophila* embryo Cell 68 201–219

Sullivan RL (1961) Linkage and sex limitation of several loci in the housefly J Heredity 52 282–286

Suzuki DT (1970) Temperature-sensitive mutations in *Drosophila melanogaster* Science 170 695–706

Tates Ad (1971) Cytodifferentiation during spermatogenesis in *Drosophila melanogaster* Ph D Thesis, University of Leiden

Tautz D Renz M (1983) An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels Anal Biochem 132 14-19

Tautz D, Pfeifle C (1989) A nonradioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback* Chromosoma 98 81-85

Technau GM Campos-Ortega JA (1986) Lineage analysis of transplanted individual cells in embryos of *Drosophila melanogaster* III Commitment and proliferative capabilities of pole cells and midgut progenitors Roux s Arch Dev Biol 195 489-498

Thummel CS, Burtis KC, Hogness DS (1990) Spatial and temporal patterns of *E74* transcription during *Drosophila* development Cell 61 101-111

Tjong S Bone LM, Whittle JRS (1985) Recessive lethal mutations within the *bithorax*-complex in *Drosophila* Mol Gen Genet 200 335-342

Tobari YN, Moriwaki D (1983) Positive correlation between male recombination and minute mutation frequencies in *Drosophila ananassae* Jpn J Genet 58 159-163

Trapitz P (1992) Analyse eines heterochromatischen Chromosoms Ansätze zur physikalischen Kartierung der Fertilitatsgene auf dem langen Arm des Y Chromosoms von *Drosophila hydei* durch Pulsfeld-Gelelektrophorese und *in situ* Hybridisierung Ph D Thesis, Institut für Genetik, Heinrich Heine Universität, Düsseldorf

Trapitz P, Wlaschek M, Bünemann H (1988) Structure and function of Y chromosomal DNA II Analysis of lampbrush loop associated transcripts in nuclei of primary spermatocytes of *Drosophila hydei* by in situ hybridization using - asymmetric RNA probes of four different families of repetitive DNA Chromosoma 96 159-170

Trapitz P, Glatzer KH, Bünemann H (1992) Towards a physical map of the fertility genes on the heterochromatic Y chromosome of *D hydei* Families of repetitive sequences transcribed on the lampbrush loops *Nooses* and *'Threads* are organized in extended clusters of several hundred kilobases Mol Gen Genet 235 221-234

Trask BJ, Pinkel D, van den Engh G (1989) The proximity of DNA sequences in interphase cell nuclei is correleated to genomic distance and permits ordering of cosmids spanning 250 kilobase pairs Genomics 5 710-717

Trask BJ Massa H, Kenwrick S, Gitschier J (1991) Mapping of human chromosome Xq28 by two-color fluorescence in situ hybridization of DNA sequences to interphase cell nuclei Am J Hum Genet 48 1-15

Traverse KL, Pardue ML (1988) A spontaneously opened ring chromosome of *Drosophila melanogaster* has acquired *He-T* DNA sequences at both new telomeres Proc Natl Acad Sci USA 85 8116-8120

Traverse KL, Pardue ML (1989) Studies of *He-T* DNA sequences in the pericentric regions of *Drosophila* chromosomes Chromosoma 97 261-271

Valgeirsdottir K, Traverse KL, Pardue ML (1990) *HeT* DNA a family of mosaic repeated sequences specific for heterochromatin in *Drosophila melanogaster* Proc Natl Acad Sci USA 87 7998-8002

Voelker RA, Kojima K (1971) Fertility and fitness of *XO* males in *Drosophila* I Qualitative study Evolution 25 119-128

Vogt P (1990) Potential genetic functions of tandem repeated DNA sequence blocks in the human genome are based on a highly conserved chromatin folding code" Hum Genet 84 301-336

Vogt P, Hennig W, Siegmund I (1982) Identification of cloned Y chromosomal DNA sequences from a lampbrush loop of *Drosophila hydei* Proc Nat Acad Sci USA 79 5132-5136

Vogt P Hennig W (1983) Y chromosomal DNA of *Drosophila hydei* J Mol Biol 167 37-56

Vogt P, Hennig W, ten Hacken D, Verbost P (1986) Evolution of Y chromosomal lampbrush loop DNA sequences of *Drosophila* Chromosoma 94 367-376

Vogt P, Hennig W (1986a) Molecular structure of the lampbrush loops nooses of the Y chromosome of *Drosophila hydei* I The Y chromosome-specific repetitive DNA sequence family ay1 is dispersed in the loop DNA Chromosoma 94 449-458

Vogt P, Hennig W (1986b) Molecular structure of the lampbrush loops nooses of the Y chromosome of *Drosophila hydei* II DNA sequences with homologies to multiple genomic locations are major constituents of the loop Chromosoma 94 459–467

von Heijne G (1987) Sequence analysis in molecular biology Treasure trove or trivial pursuit Academic Press, San Diego

Wakimoto BT, Hearn MG (1990) The effects of chromosome rearrangements on the expressionof heterochromatic genes in chromosome 2L of *Drosophila melanogaster* Genetics 125 141–154

Walsh JB (1987) Persistence of tandem arrays implications for satellite and simple sequence DNAs Genetics 115 553–567

Wapenaar MC, Kievits T, Hart KA, Abbs S Blonden LAJ, den Dunnen JT, Grootscholten PM, et al (1988) A deletion hotspot in the Duchenne muscular dystrophy gene Genomics 2 101–108

Wang F (1993) Laminin in the male germ cells of *Drosophila* Ph D Thesis, Shanghai Institute of Cell Biology, Academia Sinica, Shanghai, and Department of Molecular and Developmental Genetics, Catholic University of Nijmegen

Wang F Hanske M, Miedema K, Klein G, Ekblom P Hennig W (1992) Laminin in the male germ cells of *Drosophila* J Cell Biol 119 977–988

Wasserman M (1962) Cytological studies of the *repleta* group of the genus *Drosophila* IV The *hydei* subgroup Univ Texas Publ 6205, pp 73–83

Wasserman M (1982) Evolution of the *repleta* group In Ashburner M, Carson HL, Thompson JN (eds) The genetics and biology of *Drosophila*, vol 3b Academic Press, London New York, pp 61–139

Wheeler MR (1981) The Drosophilidae a taxonomic overview In Ashburner M, Carson HL, Thompson JN (eds) The genetics and biology of *Drosophila*, vol 3a Academic Press, London New York, pp 1–97

White MJD (1973) Animal cytology and evolution Third edition Cambridge University Press, Cambridge

Whitten MJ, Foster GG, Arnold JT, Konowalow C (1975) The Australian sheep blowfly, *Lucilia cuprina* In King RC (ed) Handbook of genetics, vol 3 Invertebrates of genetic interest Plenum Press, New York, pp 401–418

Williamson JH (1970) Ethyl methanesulfonate-induced mutants in the Y chromosome of *Drosophila melanogaster* Mutation Res 10 597–605

Williamson JH (1972) Allelic complementation between mutants in the fertility factors of the Y chromosome in *Drosophila melanogaster* Molec Gen Genet 119 43–47

Williamson JH (1976) The genetics of the Y chromosome In Ashburner M, Novitski E (eds) The genetics and biology of *Drosophila*, vol 1b, Academic Press, London New York San Francisco, pp 667–699

Willison K, Ashworth A (1987) Mammalian spermatogenenic gene expression Trends Genet 3 351–355

Wittman HG, Wittman-Liebold B (1966) Protein chemical studies of two RNA viruses and their mutants Cold Spring harbor Symp Quant Biol 31 163–172

Wlaschek M Awgulewitsch A, Bünemann H (1988) Structure and function of Y chromosomal DNA I Sequence organization and localization of four different families of repetitive DNA on the Y chromosome of *Drosophila hydei* Chromosoma 96 145–158

Wu C-I, Lyttle TW, Wu M-L, Lin G-F (1988) Association between a satellite DNA sequence and the *Responder* of *Segregation Distorter* in *D melanogaster* Cell 54 179–189

Wu Z, Murphy C, Callan HG, Gall JG (1991) Small nuclear ribonucleoproteins and heterogeneous nuclear ribonucleoproteins in the amphibian germinal vesicle loops, spheres, and snurposomes J Cell Biol 113 465–483

Wyman AR, Wertman KF (1987) Host strains that alleviate underrepresentation of specific sequences overview Methods Enzymol 152 173–180

Yamamoto M, Miklos GLG (1978) Genetic studies in heterochromatin in *Drosophila melanogaster* and their implications for the functions of satellite DNA Chromosoma 66 71–98

Yamasaki N (1977) Selective staining of Y chromosomal loops in *Drosophila hydei*, *D neohydei*, and *D eohydei* Chromosoma 60 27–37

References

Yamasaki N (1981) Differential staining of Y chromosomal lampbrush loops of *Drosophila hydei* Chromosoma 83 679-684

Young BS, Pession A, Traverse KL, French C, Pardue ML (1983) Telomere regions in *Drosophila* share complex DNA sequences with pericentric heterochromatin. Cell 34 85-94

Zacharias H, Hennig W, Leoncini O (1982) Microspectrophotometric comparison of the genome sizes of *Drosophila hydei* and some related species Genetica 58 153-157

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CURRICULUM VITAE

Peter Franciscus Renier (Ron) Hochstenbach werd geboren op 4 maart 1960 te Echt. Hij volgde de Atheneum opleiding aan het Bisschoppelijk College in Echt van 1972 tot 1978. In dat jaar begon hij met de studie biologie aan de Katholieke Universtiteit Nijmegen. Het doctoraal examen werd behaald in 1985 met als hoofdvak Genetica (Prof. W. Hennig), en als bijvakken Botanie (Prof. H.F. Linskens) en Anthropogenetica (Prof. H.-H. Ropers). Van juni 1985 tot juni 1986 was hij als drager van een Fulbright-Fellowship werkzaam in het laboratorium van Dr. M.T. Fuller, University of Colorado in Boulder, Colorado, Verenigde Staten van Amerika. Van juli 1986 tot juli 1992 was hij wetenschappelijk medewerker ("promovendus") aan de vakgroep Moleculaire en Ontwikkelingsgenetica van de Katholieke Universiteit Nijmegen. Hier werd, onder leiding van Prof. W. Hennig, het in dit proefschrift beschreven promotie-onderzoek uitgevoerd, en was hij Dr. J. Hackstein behulpzaam met de analyse van mannelijk-steriele mutanten van *Drosophila hydei*. Tevens was hij tijdens deze periode betrokken bij het onderwijs aan biologiestudenten. Sedert februari 1994 is hij post-doctoraal onderzoeker bij de vakgroep Experimentele Plantkunde van de Katholieke Universiteit Nijmegen.

STELLINGEN

1

De functionele betekenis van de *Y*-chromosomale lampenborstelloops van *Drosophila* kan alleen achterhaald worden door de eiwitten te identificeren die aan de looptranscripten zijn gebonden, dit laatste dient bij voorkeur te geschieden door gebruik te maken van mannelijk-steriele mutanten die *in trans* de morfologie van één of meerdere loops doen veranderen

2

De hypothese dat de *Y*-chromosomale lampenborstelloops een functioneel substituut vormen voor het synaptonemale complex zou een aanleiding moeten zijn om de verspreiding van zulke lampenborstelloops onder de hogere Diptera systematisch te gaan onderzoeken

3

Zoals beweerd door Zuckerkandl is een snelle evolutie van niet-coderende DNA-sequenties alleen dan te verenigen met de aanwezigheid van een functie, indien de betreffende sequentie a) een laag aandeel functionele nucleotiden bevat, en b) tijdens de evolutie vervangen is door een andere sequentie met dezelfde functie Indien dit correct is zouden de AAGAC repeats in het loop-vormende fertiliteitsgen *kl-5* van *Drosophila melanogaster* dezelfde functie moeten hebben als de veel complexere YLI, YLII of rally repeats uit het loop-vormende fertiliteitsgen *A* van *Drosophila hydei*

Zuckerkandl E (1992) J Mol Evol 32 259-271

4

De door Rice gevonden accumulatie van schadelijke mutaties door autosomen van *Drosophila melanogaster* die gedurende 35 generaties kunstmatig van recombinatie werden uitgesloten zou heel goed het gevolg kunnen zijn van inserties van retrotransposons zoals aangetoond door Steinemann voor het *neo-Y* chromosoom van *Drosophila miranda*, en, in dit proefschrift, voor het *Nooses*-gebied op het *Y* chromosoom van *Drosophila hydei*

Steinemann M & Steinemann S (1991) Chromosoma 101 169-179
Steinemann M & Steinemann S (1992) Proc Natl Acad Sci USA 89 7591-7595
Steinemann M *et al* (1993) Proc Natl Acad Sci USA 90 5737-5741
Rice WR (1994) Science 263 230-232

5

De experimenten van Pardue, Biessmann en Levis vormen de eerste bewijzen voor een specifieke functie van retroposons, namelijk het compenseren van terminale deleties van de chromosomen tijdens de premeiotische S-fase in mannetjes van *Drosophila melanogaster* Het *Y* chromosoom is mogelijk de bron voor deze telomeer-specifieke transposities

Traverse KL & Pardue ML (1988) Proc Natl Acad Sci USA 85 8116-8120
Biessmann H *et al* (1990) Cell 61 663-673
Biessmann H *et al* (1992) EMBO J 11 4459-4469
Biessmann H *et al* (1993) Chromosoma 102 297-305
Levis RW *et al* (1993) Cell 75 1083-1093

6

Uit vergelijkende studies naar de verspreiding van retrotransposons in natuurlijke populaties van *Drosophila melanogaster* kan geconcludeerd worden dat, tenminste in dit species, deze mobiele DNA-elementen als genoomparasieten bestempeld moeten worden

Charlesworth B & Langley CH in Evolution at the molecular level (Selander RK Clark AG Whittam TS eds), Sinauer Associates Sunderland Massachusetts 1991 pp 150-176

Aquadro CF in Molecular Approaches to fundamental and applied entomology (Oakeshott J Whitten MJ eds) Springer Verlag New York Berlijn Heidelberg 1993 pp 222-266

7

Het verband tussen enerzijds de - ten opzichte van de autosomen - verdubbelde dichtheid van $(CA/GT)_n$-repeats in het *X* euchromatine van *Drosophila*, en anderzijds de specifieke herkenning van het *X* euchromatine in mannetjes door antisera tegen de eiwitproducten van de genen *male-specific-lethal-one* en *maleless* en antisera tegen op lysine-16 geacetyleerd histon H4, dient nader onderzocht te worden

Paidue ML *et al* (1987) EMBO J 6 1781-1789

Huijser P *et al* (1987) Chromosoma 95 209-215

Kuroda MI *et al* (1991) Cell 66 935-947

Turner BM *et al* (1992) Cell 69 375-384

Palmer MJ *et al* (1993) Genetics 134 545-557

8

De spelling van bastaardwoorden kan slechts een compromis zijn tussen etymologie en consistentie, maar mag in geen geval verkwanseld worden aan een vereenvoudigde spellingscontrole door gedigitaliseerde tekstverwerkers

9

Iedere 53 minuten een verkeersongeval met ziekenhuisopname, en iedere 7 uur een verkeersdode het Nederlandse wegennet met de zich daarop voortbewegende weggebruikers kan moeilijk als een mensvriendelijk vervoerssysteem bestempeld worden

Nederland Het jaar in cijfers 1992, Centraal Bureau voor de Statistiek Heerlen

10

Door de als nostalgisch bedoelde herinrichting met klinkers en maaskeien van Bovenste Straat en Plats in Echt is niet alleen een verwarrende situatie ontstaan voor zowel automobilisten, (brom)fietsers als voetgangers maar is tevens het oorspronkelijke, dorpse karakter meer verstoord dan dat het is hersteld

11

Aangezien het verlagen van de luchtweerstand de belangrijkste bijdrage zal leveren aan de verbetering van het werelduurrecord fietsen, dienen toekomstige recordpogingen bij voorkeur op laaglandbanen ondernomen te worden

Nijmegen, 11 april 1994

Ron Hochstenbach

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